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The swine intestinal microbiota: localized adaptations and responses to in-feed antibiotics

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The swine intestinal microbiota: localized adaptations and responses to in-feed antibiotics

by

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ABSTRACT

After sixty years on antibiotic use in livestock production, antimicrobial resistance is an issue that cannot be ignored. While governments debate new regulations, evidence is accumulating that in-feed antibiotics are contributing to antibiotic resistance proliferation in intestinal microbes and potential pathogens. The mode of action for the growth benefits of antibiotics are not clear, but defining the intestinal bacterial communities and understanding the impact of antibiotics on it is an important link in developing viable alternatives to antibiotics. Identifying the members and functions within the bacterial communities is needed to identify niches within the intestinal tract. Here we evaluate the shifts in intestinal bacterial communities with in-feed antibiotics, which include membership and functional changes. Additionally, antibiotic-resistance genes increased in the intestinal communities after exposure to antibiotics. To evaluate localize adaptations of swine intestinal bacterial communities; the spatial distribution of bacterial communities was evaluated. Localized differences were detected and potential specialist, such mucin degraders were identified. Future research will need to direct therapies to mimic the beneficial effects of antibiotics on the host and gut bacteria while minimizing the collateral impact on health and safety.

CHAPTER 1

GENERAL INTRODUCTION

After sixty years of antibiotic use in livestock production, antimicrobial resistance is an issue that cannot be ignored. While governments debate new regulations, evidence is accumulating that in-feed antibiotics are contributing to antibiotic resistance proliferation in intestinal microbes and potential pathogens. The mode of action for the animal growth benefits of antibiotics are not clear, but defining the intestinal bacterial communities and understanding the impact of antibiotics on it is an important link in developing viable alternatives to antibiotics. Identifying the members and functions within the bacterial communities is needed to identify niches within the intestinal tract. Studies have detected shifts in intestinal bacterial communities with in-feed antibiotics, which include species and functional changes. Additionally, antibiotic-resistance genes increase in the intestinal communities after exposure to antibiotics. Concerns about antibiotic resistance in animal and human pathogens have led to research searching for alternatives to in-feed antibiotics for growth promotion and disease suppression in livestock. Alternatives such as probiotics and feed additives are promising for improving efficiencies in animal production and disease suppression. Future research will need to direct therapies to mimic the beneficial effects of antibiotics on the host and gut bacteria while minimizing the collateral impact on health and safety.

Dissertation Organization

This dissertation is organized into five chapters, including a General Introduction, three chapters to be submitted for publication, and a final Summary and Discussion with suggested directions for future studies. Chapter one is an introduction with description of the history and relevant studies on antibiotic use in agriculture. Chapter 2 examines the effects of in-feed antibiotics on swine fecal bacterial communities using a combination of 16S rRNA gene, metagenomic, and qPCR analysis. Chapter 3 is a manuscript to be submitted for publication, describes a study evaluating localized compositions of bacterial communities in the swine intestinal tract and the effects of in-feed antibiotics using 16S rRNA gene and metagenomic analysis. Chapter 4 is a description of *Cloacibacillus porcorum*, a novel mucin degrader, isolated from a pig intestine. The isolation of *C. porcorum* was part of a survey of mucin degraders along the swine intestinal tract. Chapter 5 includes general discussion and conclusions, and suggestions for future research. This dissertation uses the reference format for each chapter's respective target journal except for chapters 1 and 5, which use the ISME journal format. There are four appendices; Appendix A contains the supporting material for Chapter 2 and Appendix B includes supporting material for Chapter 3. Appendix C is a manuscript describing a study on the effects of in-feed antibiotics on bacteriophage and bacterial populations in swine fecal microbiomes. Appendix D is a list of mucin degrading bacterial isolated from the swine intestinal tract, from the survey where *C. porcorum* (Chapter 4) was isolated.

Literature review

The reality of the agriculture industry today includes antibiotics and antimicrobial resistance. Antibiotics are used to improve consistency and productivity in animals such as cows, pigs, chickens, turkeys, and fish, but decades of use has led some to question the long-term safety of antibiotic usage in animal production. Numerous studies cite the emergence of resistance genes shortly after the introduction of an antibiotic treatment (e.g. Aarestrup and Wegener 1999). Despite the clear connection between antibiotic use and resistance, the connection between antibiotic resistance in an agricultural setting and a human medical setting is uncertain. Members of the agriculture industry often point to the lack of evidence of agricultural antibiotics causing resistance in human pathogens, noting that human use is the likely driver of resistance. Both sides agree that more studies are needed to understand the effects of antibiotics in agriculture.

History of antibiotic use in agriculture

In the early 1950's the US and other countries were looking for a way to keep up with the increasing demand for food. They turned to antibiotics as a way to grow livestock healthier and fatter, because antibiotics were first shown to improve growth after chlortetracycline-containing fermentation products of *Streptomyces aureofaciens*, were fed to chickens (Jukes and Williams 1953). Governments were reaching out to farmers to teach them about the benefits of antibiotics in agriculture, including administering antibiotics in livestock feed to improve feed conversion in the animals. Antibiotics given to pigs were estimated to save as much as 20% of feed per pound of weight gain (Brock 1955).

In tandem with the growth of in-feed antibiotic practices, antibiotic resistance was detected in bacteria isolated from animals receiving antibiotics (Gyorgy 1954). Concerns were growing related to the reduced effectiveness of antibiotics over time as a growth promoter and to the development of resistant pathogens associated with animal and human disease, but ultimately benefits of reduced cost to the industry outweighed the risks (Gyorgy 1954, NRC 1956). Despite these concerns, the US was interested in expanding the use of antibiotics to include agronomy and food preservation (NRC 1956). By 1954, a quarter of all antibiotics produced in the US were used in agriculture (NRC 1956).

Sixty years later, the debate continues in the US and abroad. Concerns over the spread of antibiotic-resistance genes to human pathogens continue to drive the debate. Human foodborne pathogens with antimicrobial resistance, such as *E. coli*, *Salmonella*, and *Campylobacter*, have been isolated from livestock and poultry facilities (Addis et al 2011, Carson et al 2008, Frye et al 2011, Rao et al 2010). European nations have implemented bans on growth-promoting antibiotic uses while the US continues to search for a suitable policy. Currently, over 50% of all antibiotics sold in the US are used for agriculture, and many classes of antibiotics are used as growth promoters in farm animals (Lipsitch et al 2002). Some antibiotics used to treat disease and improve feed efficiency in animals are the same that are used to treat human treat disease (Lipsitch et al 2002). Some of these in-feed antibiotics are excreted in animals' waste, where they can persist in the environment and be unintentionally transferred to crops during fertilization (Sarmah et al 2006). These concerns have led some to question current policies permitting antibiotic use in agriculture, and others to call for improved prudence in their application (Aarestrup and Wegener 1999, Levy 1978, Levy et al 2005)

Among the groups calling for prudence, the Infectious Diseases Society of America recommended that the U.S. government adopt policies limiting the use of antibiotics in agriculture because of resistance concerns (IDSA 2010). On January, 4th, 2012 the FDA proposed a rule limiting the "off label" use of cephalosporin drugs in animal agriculture, but this did little to silence the critics (FDA 2012). A law currently proposed, Preservation of Antibiotics for Medical Treatment Act (PAMTA, H.R. 965/S. 1211), will require the FDA to review the approved antibiotics for animal feed and make determination based on implications to human health. With no compromises expected in the near future, cost-effective alternatives for in-feed antibiotics are needed.

Bacterial diversity in the GI tract

The basis for the increased feed efficiency in livestock receiving antibiotics is a debated topic, but the accepted dogma is that antibiotics are likely affecting the intestinal bacteria (microbiota) (Dibner and Richards 2005). However, antibiotic effects on the microbiota aren't easy to measure, since the mammalian gastrointestinal (GI) tract is home to a diverse population of microorganisms in a relatively inaccessible environment. As many as 1000 species are found in the gut (Eckburg et al 2005), with approximately 800 species found in the swine gut (Looft et al 2012). In humans, bacterial cells are estimated to outnumber the host cells by a factor of ten, and bacterial genes outnumber host genes by a factor of 100 (Savage 1977). This enormous diversity must first be defined before an antibiotic effect can be measured.

Interactions between the microbiota and the host are a result of a long mutually beneficial co-evolution, which confers numerous benefits on the mammalian host (Ley et al

2008). GI microbes are important for the maintenance of host health. They have important protective and metabolic functions, such as assisting the host in nutrient extraction, immune system and epithelium development, and are a natural defense against pathogens (Zoetendal et al 2004). Microbial degradation products in the GI tract supply nutrients and energy to the host, such as vitamins, volatile fatty acids, growth factors, and under normal conditions, interactions are the result of a subtle balance that promotes symbiosis (Savage 1977). Studies using germ-free animals have shown that colonization with commensal bacteria affects the expression of host genes, including those associated with mucosa maturation, nutrient uptake, metabolism, and angiogenesis (Hooper and Gordon 2001, Hooper et al 2001). Germ free mice require 30% more calories than conventional mice to maintain their weight, because germ free animals don't have a microbial community to help extract nutrients from food (Wostmann et al 1983). Understanding the interactions between the gut microbiome and the host will inform decisions on antibiotic usage and improve animal health.

Host mucosa and immune system

Bacteria that colonize the GI mucosa are particularly important for animal health because they are in direct contact with the host (Rosebury 1962). The mucosa is a layer of epithelium with connective tissue and smooth muscle underneath and is responsible for nutrient absorption. Most interactions between commensal bacterial and the host mucosa are neutral or beneficial, but some interactions may lead to disorders such as Crohn's disease or ulcerative colitis (Joossens et al 2011). Altered mucosal-associated bacterial communities have been shown to be linked to inflammatory bowel disease in humans (Campieri and

Gionchetti 2001, Sartor 2008), differ between the ileum to the colon (Eckburg et al 2005), and may even be gender specific (Aguirre de Carcer et al 2011).

The mucosa has many microenvironments for bacterial-host interactions, in part because it contains many folds and villi that protrude into the interior, or lumen, of the GI tract and invaginations called crypts that increase the surface area for nutrient absorption. The mucosa is composed of many specialized cells that have digestive and protective functions. Enterocytes are intestinal absorptive cells found on the villi and contain microvilli, which further increase the surface area of the mucosal surface for absorption. While increasing the surface area for digestion, these folds also facilitate interactions between the mucosa and the indigenous bacteria (Nataro 2005). Paneth cells are found at the bottom of the crypts and secrete the most of the antimicrobial peptides produced in the small intestine. These peptides are secreted when bacteria are detected and include α-defensins, lysozyme, and RegIIIγ. In addition to this range of antimicrobial compounds, mucosally-associated bacteria also have to deal with a gradient of oxygen that is diffusing from the underlying tissue, which is harmful to obligate anaerobes (Bornside et al 1976). A thick mucus layer is generated by columnar epithelial cells (goblet cells), serving to limit the contact with and penetration by the gut bacteria within the mucosa. The mucus layer also protects the bacteria from the host's defenses mentioned above, thus contributing to the balance of the host/microbial relationship.

The immune system is also an important factor in maintaining the balance between the host and its microbiota. Peyer's patches are round lymphoid follicles covered by specialized epithelial cells called microfold cells (M-cell). M-cells sample antigens directly from the lumen and deliver them to antigen-presenting cells, resulting in B-cells and memory

cell stimulation. Plasma cells (B cells) and T cells can be found in the follicles of Peyer's patches where they encounter foreign antigens. Intestinal microbes are important for lymphocyte development such as, B-cell class switching, Th17 effector T-cell development, and induction of T-regulatory cells (Duerkop et al 2009). The innate and adaptive immune systems work together to detect and influence the diverse microbial populations associated with the intestinal mucosa.

Bacterial specialists in the GI tract

In addition to looking at bacterial species diversity, important research focuses on functional subdivisions of bacteria in the gut ecosystem. Functional redundancy or niche specificity conferred by bacterial specialists can be shared across unrelated bacterial species but usually indicates important functions in the gut. Specific bacterial metabolic activities and fermentation end-products have been shown to benefit the host and are also targets to manipulate for improved efficiencies in animal production. Mammalian hosts have learned to tolerate many beneficial specialists, even possessing specific cellular transporters of bacterial fermentative products (Cresci et al 2010). The colonic mucosal epithelium in particular depends on bacterially-derived nutrients diffusing from the lumen for energy (Bengmark 2000). Other specialists benefit the host by simply occupying available niches on the mucosa, preventing colonization of pathogens (Poole et al 2009, Thomas et al 2011). Because of these interactions, bacteria that provide the host with essential nutrients or are in contact with the mucosa are the focus of current research.

Lactic acid bacteria

An important group of gut specialists in both livestock and humans are the lactic acid bacteria (LAB). LAB are Gram positive, facultative anaerobes that are able to ferment sugars to lactic acid via fermentative pathways (Bernardeau et al 2008), and consist of over 400 recognized species (Euzeby 1997). Often available in dairy, fermented foods, or as a probiotic, LAB are known to modulate the intestinal tract to improve mucosal barrier function, antagonize pathogens, and reduce the severity and duration of viral diarrhea (Heyman 2000). Additionally, LAB are effective at improving weight gain and minimizing infections in livestock, even in the absence of antibiotics (Abe et al 1995, Signorini et al 2011). One study showed that the LAB *Lactobacillus casei* inhibited the ability of *Pseudomonas aeruginosa* and *Listeria monocytogenes* to colonize mice by stimulating the host immune system, thereby increasing the numbers of macrophages that prey on the pathogens (Driessen and De Boer 1989). Additionally, LAB may have anti-carcinogenic effects, reducing microbial enzymes responsible for converting procarcinogens to carcinogens (Gorbach and Goldin 1990). Several studies have reported a reduction in LAB after antibiotic treatment, which may be an unintended side-effect of antibiotic use (Allen et al 2011, Antonopoulos et al 2009, Hill et al 2010, Ichinohe et al 2011). As potential antagonists of intestinal pathogens and as producers of small molecules important for host health, LAB's role in animal health is vital.

Butyrate-producing bacteria

Butyrate production by microorganisms in the gut has been shown to benefit the health of the host by contributing a major portion of the caloric intake in ruminants, pigs, and

other livestock. Butyrate is absorbed and used by the colonic epithelium as an energy source, acting as a growth-promotant by stimulating epithelial cell proliferation (Sakata and von Engelhardt 1983, Sunkara et al 2011). Additionally, butyrate stimulates mucin synthesis along the intestinal mucosa, which improves the barrier against pathogens and is considered an indication of gut health (Brown et al 2011, Finnie et al 1995, Hatayama et al 2007).

Mucin-degrading bacteria

Adaptations for mucosal colonization are important to identify when evaluating bacterial roles in animal health, and one such adaptation could be mucus degradation. As mentioned previously, the intestinal mucosa is covered by a layer of mucus, which protects the underlying epithelium from damaging agents and limits microbial penetration of the mucosa. Mucus is mostly water and mucin, which is the viscous part of the mucus. Mucins are glycoproteins with many carbohydrate side chains connected to a main protein by O-glycosidic links (Nataro 2005). Most mucins are secreted (gel-forming mucins) but some have trans-membrane regions and are bound to the membrane of epithelial cells (membrane-associated mucins) (Ouwehand et al 2005). One of the major functions of mucins is to prevent bacterial attachment to the surface of epithelial cells by offering recognition sites similar to the epithelial cell surface (Nataro 2005). The mucus layer is stratified and can be 150 µm thick. The majority of mucosally associated bacteria are able to penetrate the outer mucus layers but never reach the mucosal epithelium. Additionally, mucus and epithelial cells are constantly sloughed off to combat colonization of the mucosa. Associations of microbes with mucus is not well understood (Derrien et al 2008), but a diverse population of mucus-degrading bacteria have been identified and are associated with the mucus layer

(Rozee et al 1982). Mucin-degrading activity has been linked to pathogenesis in some microbes (Campieri and Gionchetti 2001). Not all mucin degraders are pathogens, and evaluating this subpopulation for effects on animal health and potential therapeutic uses is an important step towards targeting alternatives for in-feed antibiotics.

How antibiotics in feed lead to improved feed efficiencies

In order to develop viable alternatives to antibiotics, we have to know how the in-feed antibiotics are functioning to improve feed efficiency. The mode of action for antibiotics is not clear, but mechanisms may include a reduction in total bacterial load, increased nutrient absorption by the hosts, or community modeling by favoring non-antagonistic or beneficial bacteria and functions (Butaye et al 2003). The most accepted dogma towards antibiotic use in livestock production is that antibiotics improve feed efficiency by knocking down bacterial load. This decreases the energy expenditure by the host because the host's immune system is constantly maintaining/controlling gut microbes (Jukes and Williams 1953). Reduced mucosal inflammation due to a reduced bacterial load may be a specific response that reduces the energy cost to the host, thus leaving the surplus calories for weight gain. The hypothesis, therefore, is that fewer bacteria in the gut decreases the amount of energy spent on immunity, allowing that energy to divert into growth. Additionally, the reduced bacterial load (reduced bacterial metabolism) may lead to more nutrients being available for host absorption (Hardy 2002). In addition to improving animal growth, antibiotics decrease morbidity and mortality due to clinical and subclinical diseases (Solomons 1978).

Collateral effects of in-feed antibiotics on the gut microbiota

On disease susceptibility

The benefit of improved feed efficiency by feeding antibiotics is not without a cost. Increased disease susceptibility may be a collateral effect of antibiotic use. For example, eliminating commensals with broad-spectrum antibiotics can result in a reduction of host-produced antimicrobial molecules in the intestinal mucosa, increasing susceptibility to antibiotic-resistant bacteria and pathogens (Brandl et al 2008, Hill et al 2010).Treatment with the antibiotics streptomycin and metronidazole also leads to increased infections because they alter the mucus layer, thereby weakening its barrier function (Wlodarska et al 2011). Antibiotic treatment has also been shown to predispose mice for Salmonella infections. *Salmonella* populations increased after withdrawal from an oral antibiotic regimen because antibiotics perturbed the intestinal microbial ecosystem (Croswell et al 2009, Sekirov et al 2008). The undesired collateral effects of antibiotics may not always be evident, but should be included in the cost-benefit analysis of agricultural antibiotics.

On beneficial microbes and functions

Numerous effects of antibiotics on the intestinal microbial membership and functions have been studied in recent years. Impacts on microbial community membership are measured using conserved phylogenetic makers (e.g. the 16S rRNA gene sequence) to make taxonomic assignments and compare communities. Functional studies often look at the functional capacity of the community (gene content –metagenome [studies –metagenomics]), but can include gene expression and products as well.

Understanding antibiotic-induced shifts in the membership (phylotypes) are an essential first step towards identifying which bacterial groups are of functional importance. In humans, the administration of ciprofloxacin affected the abundance of the majority bacterial taxa in the gut, resulting in decreased richness, diversity, and evenness. After a four week withdrawal period, the microbiota of the treated individuals resembled the pretreated state, but several taxa didn't recover (Dethlefsen et al 2008). This is concerning because loss of specific commensal bacteria may impact host health. For example, the bacterium *Oxalobacter formigenes* is lost after antibiotic treatment in humans, and can be difficult to re-establish. *O. formigenes* degrades oxalate, the accumulation of which results in the formation of calcium oxalate kidney stones (Duncan et al 2002). In agriculture, a study of swine given sub-therapeutic levels of ASP250 (chlortetracycline, sulfamethazine, and penicillin) showed an increase in populations of commensal *E. coli* and a reduction in *Streptococcus* spp. (Allen et al 2011, Looft et al 2012). The ability of the microbiota to recover after antibiotic treatment differs by the antibiotic regimen administered, and the impact of a failed microbial recovery on the host is unknown.

Alterations in bacterial membership have important implications on the functional capacity of the microbiota, which in turn may confer a health advantage on the host. For example, particular changes in the bacterial communities, such as with obesity or antibiotic exposure, may be associated with improved energy harvesting capabilities of the gut microbiota (Ley et al 2005, Ley et al 2006, Looft et al 2012, Turnbaugh et al 2006). Changes in the relative abundance of *Bacteroidetes* and *Firmicutes* in obese individuals also reflects functional shifts towards increased energy harvesting within the microbial communities (Ley et al 2006, Turnbaugh et al 2006). Perhaps the growth-promoting effects in animals fed

antibiotics are analogous to the changes in metabolic potential of the intestinal microbiota in obese individuals, which could be improving feed efficiency (Looft et al 2012, Turnbaugh et al 2006).

In addition to metagenomic studies, analyses of community-wide effects of antibiotics have included community metabolite studies (metabolome). Antunes et al. (2011) showed that most of the metabolites detected in the communities with exposure to antibiotics, shifted in abundance, when compared to the non-treated communities. Affected host metabolic pathways included those critical for animal physiology, including bile acid, eicosanoid, and steroid hormone synthesis. Functional studies are important because they directly address questions about what the bacteria in the community are doing and how that relates to host health. Functional redundancy across bacterial species may mask significant functional changes with antibiotic usage if only phylotype shifts are considered.

The GI antibiotic resistome

Of the functional genes to change with antibiotics, those associated with antibiotic resistance mechanisms would be the most intuitive because of the competitive advantage they confer. The antibiotic “resistome” is the total reservoir of resistance determinants in a microbial community (D'Costa et al 2006). An enormous diversity of antibiotic resistance genes have been detected in environmental and intestinal ecosystems (Allen et al 2010, Sommer et al 2009), and these may persist even with the absence of antibiotics (Salyers and Amabile-Cuevas 1997). Indeed, the microbiota of healthy humans and swine are antibiotic resistance reservoirs, harboring diverse and numerous resistance genes (Sommer et al 2009). Swine receiving sub-therapeutic (growth promoting) antibiotics (ASP250), had increases in

the abundance and diversity of antibiotic resistance genes, including resistance to antibiotics not administered (Looft et al 2012). Resistomes such as these may contribute to the preservation and expansion of antibiotic resistance determinants, particularly in the presence of antibiotic selection.

The strong selective pressure of antibiotic use as part of livestock management is in part responsible for the numerous resistance genes found in livestock microbiota, but antibiotic resistance continues to persist even after antibiotics are no longer used. Despite an absence of antibiotic exposure, organically raised pigs maintain a diverse number of tetracycline resistance genes, many plasmid encoded (Kazimierczak et al 2009). In another study, chlortetracycline resistant bacteria in feral swine feces were over 1,000-fold fewer, when compared to organically raised pigs (Stanton et al 2011). In the absence of antibiotics, piglets pre-colonized with antibiotic susceptible strains of *Megasphaera elsdenii* lost the susceptible strains, in favor of the mother's resistant *M. elsdenii* strains (Stanton and Humphrey 2011). These findings point to lasting effects that antibiotic use with livestock production has, and even with elimination of antibiotics and attempts to pre-colonize with susceptible strains, resistanct bacterial strains persists.

One collateral effect of antibiotics is antibiotic-induced dissemination of resistance genes between bacteria. In some cases, antibiotics actually stimulate the transfer of resistance genes on mobile elements between intestinal bacteria (Salyers and Shoemaker 1992). When resistance genes are on plasmids, they can spread quickly, even between distantly related bacteria or pathogens (Gotz et al 1996, Ochman et al 2000). Mucosal inflammation can also lead to an increase in horizontal gene transfer between *Salmonella* and *E. coli* (Stecher et al 2012). Antibiotic resistance genes have been identified in bacteriophages in livestock feces,

which may facilitate the transfer of antibiotic resistance genes to potential animal or human pathogens (Allen et al 2011). Several studies have demonstrated that antibiotics used in livestock production actually induce phage lysis of their bacterial host and facilitate gene transfer (Maiques et al 2006, Stanton et al 2008, Ubeda et al 2005). The potential human pathogens *Enterococcus faecalis* and *E. faecium* have been isolated from broiler chicken and turkey flocks with resistance to bacitracin, erythromycin, flavomycin, gentamicin, kanamycin, lincomycin, quinupristin- dalfopristin, streptomycin, tetracycline, and tylosin; various combinations of these resistance genes were identified on plasmids (Tremblay et al 2011). The selective pressure of in-feed antibiotics increases the risk of pathogens with resistance to multiple antibiotics.

Livestock animals may be acting as living test-tubes, facilitating gene transfers between human and animal isolates of related species. Methicillin-resistant *Staphylococcus aureus* has been seen in livestock since the 1970's. Long considered a livestock pathogen, it is now becoming a problem for human health (Devriese et al 1972, Price et al 2012). Genome-wide surveys of *S. aureus* strains suggest that human-associated methicillin-susceptible *S. aureus* have colonized livestock, where the strains acquired resistance to methicillin and tetracycline (Price et al 2012). In the mouse colon, vancomycin resistance genes were conjugatively transferred from porcine to human *Enterococcus faecium*, again suggesting that animal-associated bacteria may serve as an antibiotic resistance reservoir for human bacterial species (Moubareck et al 2003). Antibiotic resistance itself doesn't make a pathogen, but the transfer of resistance genes to human or animal pathogens may be more common than previously estimated.

Alternatives to in-feed antibiotics

Concerns about antibiotic resistance make finding alternatives for the growth-promoting and therapeutic uses of agriculture antibiotics essential. An all-out ban on antibiotics in agriculture may not be the best approach. In Europe after in-feed antibiotics were banned in livestock production, disease occurrences increased while production efficiencies decreased (Ferber 2003). Some have suggested that antibiotics in animal production should only have a role in disease treatment and that feeding prophylactically is a poor strategy (Hardy 2002). Viable alternatives are needed to maintain efficiency standards and minimize disease.

Antibiotic alternatives for improved feed efficiency

Improving the nutrient composition of animal feed and feed additives are important ways to improve weight gain in livestock. Prebiotics, which are substrates added to feed to stimulate the production of gut microbial nutrients, usually include dietary fibers and complex proteins (Bengmark 2000). Extensive research has been performed on certain feed additives, including organic acids, biomos, anhydrotetracycline, as well as improving feed digestibility and nutrient availability. In-feed organic acids such as lactic and formic acid improve pig immune response and reduce *Salmonella* in swine feces (Lee et al 2007, Walsh et al 2012, Willamil et al 2011). Folic acid is crucial for DNA and methionine metabolism during pregnancy and lactation in livestock (McNulty et al 1993). Fermented liquid diets have also been looked at as an alternative to antibiotics for growth improvements. Liquid feed is fermented with LAB, which can improve performance in pigs by easing the transition

from the sow milk to solid feed. Fermented feed is acidic, limiting food spoilage, lowering stomach pH, and resulting in lower pathogen survival (Missotten et al 2010).

Augmenting the functionality of butyrate-producing bacteria in the gut is currently an important focal point for prebiotics. Prebiotic substrates that are efficiently converted to butyrate may be added to livestock diets to stimulate butyrate production. Starches resistant to digestion by the host have been shown to be the most powerful butyrogenic substrate in the intestinal tract, increasing butyrate production by up to 25% (Bird et al 2000, Brouns et al 2002). Resistant starches could be used to affect the microbial community by stimulating butyrate-producing organisms. Selecting for lactate producers may also increase butyrate production in the gut because butyrate producers can use free lactate to produce butyrate as an end product (Silvi et al 1999). Prebiotic feed additives such as these stimulate particular gut bacteria to target functions that benefit the host, achieving similar results to antibiotics.

Antibiotic alternatives for disease treatment and prevention

Antibiotics are not the only approach for reducing potential pathogens and subclinical infections in animal agriculture. Methods using probiotics and prebiotics to competitively exclude pathogens are gaining in popularity (Hardy 2002). Bacteria that are antagonistic to pathogens, such as LAB, make interesting targets for reducing the pathogen load (Heyman 2000, Mappley et al 2011). Probiotics have been effective in reducing *Salmonella*, *E. coli*, *Clostridium perfringens, Campylobacter jejuni*, and many other livestock pathogens (Chu et al 2011, Han et al 2011, Meng et al 2010, Santini et al 2010). Many bacterial species have been evaluated as potential probiotic strains for their beneficial characteristics including; *Lactobacillus, Streptococcus, Bifidobacterium, Bacillus* and yeasts (Dunne et al 2001, Guo et

al 2006). Several traits are important in a good probiotic strain: resistance to stomach acids and bile, the ability to colonize the intestine, produce essential nutrients, and antagonize pathogens. *Bacillus* species have been used in pigs with some success, resulting in an increased growth rate and decreased mortality (Davis et al 2008, Kyriakis et al 1999).

A combination of these practices with other improvements may have an additive effect. Genetic selection of disease resistance in livestock should improve the herd health, negating the use of antibiotics. Additionally, environmental management could be improved. A reason that antibiotics are needed to combat disease is the close living confinements in which livestock are raised. Smaller, segregated animal groups would limit disease transmission. To be viable in the field, alternatives need to be effective at both performance and disease suppression at a cost comparable to antibiotics.

Summary

Animals have an intricate and complex relationship with the bacterial milieu in their guts. Intestinal microbes provide many important protective and metabolic functions, including the extraction of nutrients from otherwise indigestible compounds. Interactions along the intestinal mucosa are particularly important for animal health and simulate the host immune system (Ashida et al 2012). Commensal microbes even have the potential to be prescribed biological supplements for use as therapeutic agents (Hooper and Gordon 2001). The delicate balance of beneficial gut bacteria is modified by antibiotics. It is important to understand the mechanisms by which antibiotics are shaping the microbiota, especially regarding the maintenance and acquisition of resistance genes. Knowledge of shifts in the microbiota during antibiotic regiments can inform strategies for alternative to antibiotics

while maintaining efficiencies in livestock production. There is significant potential for pre- and probiotics as alternatives for antibiotics.

Future directions with host-bacterial interactions in the gut

Host-microbe relationships that benefit the host will continue to be the focus of future research improving animal health and production. Understanding intestinal bacterial diversity and niches occupied by commensals will inform studies targeting specific groups. New molecular technologies have improved our ability to deeply sample (sequence) these communities, allowing researchers to test hypotheses that were previously not possible. Data on gene expression and the resulting products will give valuable insights into the functions and interactions between bacteria and the host. Host gene expression is the other side of this relationship, and is important when evaluating bacterial roles on animal health. In all, these techniques will start to fill in the details of how intestinal bacteria influence animal health, particularly regarding the improvement of performance in the absence of antibiotics.

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CHAPTER 2. IN-FEED ANTIBIOTIC EFFECTS ON THE SWINE INTESTINAL MICROBIOME

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Looft T, Johnson TA, Allen HK, Bayles DO, Alt DP, Stedtfeld RD et al (2012). In-feed antibiotic effects on the swine intestinal microbiome. *Proceedings of the National Academy of Sciences* 109:1691-6. I designed this experiment with Thaddeus Stanton. Additionally, I collected the samples, extracted DNA, performed all 16S rRNA gene PCR reactions, prepared amplicon and metagenomic libraries for sequencing, analyzed the 16S rRNA data and metagenomic data, and wrote this manuscript with Heather Allen and Tim Johnson.

Abstract

Antibiotics have been administered to agricultural animals for disease treatment, disease prevention, and growth promotion for over 50 y. The impact of such antibiotic use on the treatment of human diseases is hotly debated. We raised pigs in a highly controlled environment, with one portion of the littermates receiving a diet containing performance-enhancing antibiotics [chlortetracycline, sulfamethazine, and penicillin (known as ASP250)] and the other portion receiving the same diet but without the antibiotics. We used phylogenetic, metagenomic, and quantitative PCR-based approaches to address the impact of antibiotics on the swine gut microbiota. Bacterial phylotypes shifted after 14 d of antibiotic treatment, with the medicated pigs showing an increase in *Proteobacteria* (1–11%) compared with nonmedicated pigs at the same time point. This shift was driven by an increase in *Escherichia coli* populations. Analysis of the metagenomes showed that microbial functional genes relating to energy production and conversion were increased in the antibiotic-fed pigs. The results also indicate that antibiotic resistance genes increased in abundance and diversity in the medicated swine microbiome despite a high background of resistance genes in nonmedicated swine. Some enriched genes, such as aminoglycoside O-phosphotransferases, confer resistance to antibiotics that were not administered in this study, demonstrating the potential for indirect selection of resistance to classes of antibiotics not fed. The collateral effects of feeding subtherapeutic doses of antibiotics to agricultural animals are apparent and must be considered in cost-benefit analyses.

Introduction

Antibiotics are the most cost-effective way to maintain or improve the health and feed efficiency of animals raised with conventional agricultural techniques (1, 2). In addition to improving feed efficiency, antibiotics are commonly given to livestock, poultry, and fish for disease treatment and prevention. The sum of agricultural antibiotic use reportedly accounts for as much as half of all antibiotics produced in the United States (3). Despite the clear benefits of antibiotics to agriculture, liberal antibiotic use combined with rapid and widespread emergence of both animal and human pathogens resistant to multiple antibiotics has led some to question the prudence of current antibiotic use (4, 5). Studies of environmental and intestinal microbial communities reveal enormous diversity of antibiotic resistance genes (6–8). The addition of antibiotics to feed introduces a selective pressure that may lead to lasting changes in livestock commensal microorganisms. Furthermore, reservoirs of antibiotic resistance genes have been shown to be stable in bacterial communities, even in the absence of antibiotics (9–12). A central concern of increased abundance of antibiotic resistance is the transfer of resistance to pathogens (13). As a result, the Food and Drug Administration recently released a draft guidance recommending restrictions on the use of antibiotics in animal agriculture (14). The Infectious Diseases Society of America testified before a Congressional subcommittee in support of such limitations (15).

Bacteria that inhabit the gastrointestinal tract of animals are important for the maintenance of host health. The intestinal microbiota assists the host in nutrient extraction, immune system and epithelium development, and are a natural defense against pathogens (16). Contrary to these benefits, the gut microbiota may antagonize future disease treatment by facilitating the dissemination of resistance alleles across distantly related organisms. For

example, commensal bacteria of the human colon harbor antibiotic resistance genes and can transfer these genes to pathogens (17, 18). In fact, horizontal gene transfer is largely the cause of multidrug resistance in Gram-negative bacteria (19). With the identification of antibiotic resistance genes in commensal bacteria in the human food-chain (20–22), the role of the gut microbiota as a reservoir of resistance genes for animal and food-borne pathogens needs to be explored.

Valuable insights have been gained by culture- and PCR-based approaches to study narrow groups of bacteria or genes, such as erythromycin resistance in swine isolates (23); however, the comprehensive effects of daily feeding of subtherapeutic doses of antibiotics on livestock microbiotas have not been studied. We therefore sought to extensively evaluate the effects of in-feed antibiotics on the entire gut microbiota. Phylotyping, metagenomic, and parallel quantitative PCR (qPCR) approaches were used to track changes in microbial membership and encoded functions, enabling the detection of so-called “collateral” effects of antibiotics (i.e., effects outside of the intended growth promotion and disease prevention). These collateral effects included increases in *Escherichia coli* populations and in the abundance of certain antibiotic resistance genes.

Piglets were birthed at the National Animal Disease Center in Ames, IA, and housed together in highly-controlled, decontaminated rooms to avoid cross contamination among the medicated animals, nonmedicated animals, and other resident barn animals. Neither the piglets nor the sow were exposed to antibiotics before the study. This design was to ensure that the inoculum for the piglets would come horizontally from their mother, minimizing variability so that effects of antibiotic treatment could be detected. At 18 wk of age, one group of littermates received ASP250 feed (medicated) and the other received the same but

unamended feed (nonmedicated) for 3 wk. ASP250 is an antibiotic feed additive containing chlortetracycline, sulfamethazine, and penicillin that is commonly given to swine for the treatment of bacterial enteritis and for increased feed efficiency. Fecal samples were collected just before treatment (day 0), and after 3, 14, and 21 d of continued treatment. Day 0 samples were used to describe the swine intestinal microbiome before antibiotic treatment period.

Results

Shifts in Community Membership with ASP250.

We collected 133,294 sequences of the V3 region of the 16S rRNA gene from a total of 12 fecal samples. Data from pigs of the same treatment and sampling date were grouped to appraise an antibiotic effect on community membership. As reported for a mammalian intestinal environment (24), and recently in a swine metagenome (25), the majority of classifiable sequences (75–86%) belonged to the Bacteroidetes, Firmicutes, and Proteobacteria phyla (Supplementary table A1). Of the Bacteroidetes, the Prevotella genus was consistently abundant, as was shown to be a feature of the swine microbiome (25). The Bray-Curtis index was calculated for all sample combinations and an analysis of similarities (ANOSIM) was performed. A nonmetric multidimensional scaling (NMDS) plot of these data indicated divergence of the day 14 samples from the day 0 samples ($P < 0.01$), and the medicated microbiome diverged from the nonmedicated ($P < 0.05$) (Figure 2.1A), demonstrating changes in microbial community membership over time and with treatment.

Specific changes in the microbial community associated with ASP250 treatment included a decrease in the abundance of Bacteroidetes, along with members of Anaerobacter, Barnesiella, Papillibacter, Sporacetigenium, and Sarcina genera. Members of the Deinococcus-Thermus and Proteobacteria phyla increased with ASP250 treatment as well as Succinivibrio and Ruminococcus genera (Supplementary table A1). The increase in Proteobacteria abundance with in-feed ASP250 was particularly striking: from 1% of the population in nonmedicated animals to 11% of the population with antibiotic treatment (Figure 2.1B). Specifically, E. coli populations were the major difference between medicated and nonmedicated animals, comprising 62% of the Proteobacteria in medicated animals (Figure 2.1C). The increase in E. coli was confirmed in the metagenomic data (Figure 2.1D) and by qPCR targeting the *uidA* gene of *E. coli* ($P < 0.05$). A separate study using 12 pigs similarly treated but with analysis by culture-based techniques further established that swine fed ASP250 have an increased *E. coli* population at 14 d posttreatment, showing a 20- to 100-fold greater *E. coli* abundance in medicated than nonmedicated swine (Supplementary figure A1).

Shifts in Functional Gene Abundance with ASP250.

DNA samples from the feces of nonmedicated and medicated pigs at days 0 and 14 were isolated, and samples of like treatment and sampling date were pooled for pyrosequencing. Metagenome sequences (1,202,058 total) were analyzed in MG-RAST for SEED subsystems (26), and in-house for clusters of orthologous groups (COGs). All metagenomes showed functional stability over time by both COG and subsystem analyses (Supplementary figure A2). The most abundant SEED subsystem of known function was

carbohydrate metabolism, mirroring what was previously reported for the swine metagenome (25). A statistical analysis of COGs revealed shifts in microbial community functions with ASP250: the medicated metagenome contained 169 COGs that were significantly more abundant than in the nonmedicated metagenomes (Supplementary table A2). Three COGs (0477, permeases of the major facilitator superfamily; 1289, predicted membrane protein; 3570, streptomycin 6-kinase) contain swine metagenomic genes that are annotated as resistance genes in the antibiotic resistance gene database (ARDB). Three of the COGs with the lowest P value (3188, 3539, and 3121) contained genes related to P pilus assembly, and additionally among the statistically significant COGs are transposases (0675, 1662, and 4644).

To identify themes among differentially represented COGs between the medicated and nonmedicated metagenomes, COGs of Supplementary table A2 were clustered by their respective COG category. Only one COG functional category, energy production and conversion (C), was found more frequently ($P < 0.05$) in the medicated metagenome than in the nonmedicated metagenomes (Supplementary table A3).

Pervasive Antibiotic Resistance in the Absence of Antibiotic Exposure.

The discovery that resistance-related COGs fluctuated with antibiotic treatment led to further scrutiny of the metagenomes by BLAST against the ARDB (27). All metagenomes, regardless of antibiotic treatment, harbored sequences similar to diverse antibiotic resistance genes representing most mechanisms of antibiotic resistance: efflux pumps, antibiotic-modifying enzymes, and modified or protected targets of the antibiotic (Figure 2.2A). This analysis detected 149 different resistance genes in the day 0 metagenomes.

The finding of diverse fecal antibiotic resistance genes in the nonmedicated metagenomes was supported by parallel qPCR analysis. A rich array of 57 resistance genes was detected at least once in the swine fecal samples by qPCR. Samples from nonmedicated animals showed a total of 50 different resistance genes, but few were shared between animals: only five [*ermA*, *ermB*, *mefA*, *tet*(32), and *aadA*] were detected in 66% of the samples and none were found in more than 80% of the samples. No enrichment of these genes was observed in the medicated animals, even though *tet*(32), a ribosomal protection protein, is known to confer resistance to an administered antibiotic (tetracycline). Samples from medicated animals yielded more homogenous resistance gene diversity: 38 genes were detected in at least one medicated sample, 19 were detected in 66% of samples, and 10 [*mefA*, *ermA*, *ermB*, *tet*(32), *tet*(O), *aadA*, *aph*(3′)-ib, *bcr*, *acrA*, and *bacA*] were detected in at least eight of nine of the samples.

qPCR and Metagenomic Analyses Reveal Shifts in Resistance Gene Richness and Abundance in Medicated Pigs.

Statistical analysis of the ARDB results showed 23 genes to be differentially represented in the medicated and nonmedicated metagenomes (Table 2.1). The 20 genes that were more abundant in the medicated metagenome were associated with efflux, sulfonamide resistance, and aminoglycoside resistance, the latter of which represents resistance to a class of antibiotics not present in ASP250 (Table 2.1).

The qPCR results mirrored the metagenomic analysis, revealing six resistance-gene types with statistically significantly greater abundance in the medicated animals than in the nonmedicated animals ($P < 0.05$): tetracycline efflux pumps, class A β-lactamases,

sulfonamide resistance genes, aminoglycoside phosphotransferases, and two types of multidrug efflux (Figure 2.2B and Table 2.1). No statistical difference in abundance was found for these six resistance gene types between the medicated and nonmedicated microbiomes on day 0 (Figure 2.2B), suggesting that in-feed ASP250 caused the effect. Resistance-gene abundance increased most dramatically in the 3- and 14-d samples (Supplementary figure A3), indicating that antibiotic treatment induced a rapid shift in the abundance of resistance genes.

ASP250 treatment increased the diversity of resistance gene types as detected by qPCR [Shannon indices 1.4 (medicated) and 0.8 (nonmedicated); $P = 0.04$]. A t test comparing the mean number of resistance genes in the metagenomes at day 14 to the corresponding nonmedicated metagenome confirms this result ($P < 0.05$). Additionally, the structure of the resistance-gene communities (β-diversity) was altered by antibiotic treatment, as determined by a two-way ANOSIM ($P < 0.01$) of Bray-Curtis measures; however, the comparison R-value was 0.25, indicating that the degree of separation is limited. Nevertheless, resistance gene diversity converges with ASP250 treatment, presumably because of the selective pressure of the antibiotics (Figure 2.2C). Taken together, these results show that feeding antibiotics increases the diversity of resistance genes within an individual sample and homogenizes that diversity between treated samples.

Discussion

We assessed the effect of ASP250 on the swine antibiotic resistome using phylotype, metagenomic, and qPCR approaches. The results show that the swine microbiome harbors diverse resistance genes even in the absence of selective pressure. Five genes in particular

were detected at high frequency in both the medicated and nonmedicated microbiomes. These genes could represent a core antibiotic resistome for this cohort of swine. Indeed, it was suggested that *tet*(32) is abundant in farm animals (28), and our data support that conclusion for swine. The constant selective pressure of 50 y of in-feed antibiotics appears to have established a high background level of resistance in the swine microbiome.

Antibiotic treatment caused a detectable increase in the abundance of resistance genes even above the high background of resistance, and many of these were likely enriched because of direct interaction with the antibiotics in ASP250. For example, sulfamethazine presumably selected for the sulfonamide resistance genes *sul2* or *sul1*, present in eight of the nine medicated samples. Additionally, class A β-lactamases were overrepresented in the medicated animals and confer resistance by cleaving such β-lactam antibiotics as penicillin. Many of the other enriched resistance genes function by exporting chemicals. Such efflux includes but is not limited to antibiotics and may allow bacteria that lack specific resistance genes to survive antibiotic pressure. Multidrug efflux is frequently associated with the medically alarming issue of multiple-drug resistance and can be found on mobile genetic elements (29). In addition to the effects on specific gene families, in-feed antibiotics homogenized the richness of resistance genes among individuals over time. The breadth of the current study enabled the visualization of this intriguing phenomenon despite the tremendous resistance gene heterogeneity across samples.

One type of resistance, the aminoglycoside O-phosphotransferases, increased in abundance with in-feed ASP250, although it does not confer resistance to the antibiotics therein. This finding suggests an indirect mechanism of selection, perhaps by co-occurrence on mobile elements conferring resistance to ASP250 antibiotics. Ten of the 13

phosphotransferases identified in the medicated swine metagenome are homologous (7 of 10 have 100% amino acid identity) with the streptomycin phosphotransferase on the pO86A1 plasmid in *E. coli* O86:H- (accession number YP_788126). Resistance genes aggregate on plasmids in response to selective pressure (30), and pO86A1 carries at least two other resistance genes (accession number NC_008460). This congregation of resistance genes on mobile genetic elements could offer a fitness advantage to a bacterium living in the constant presence of antibiotics. However, this would be an undesirable collateral effect of in-feed antibiotics because these resistance gene clusters could be transferred to *E. coli* or other potential human pathogens in the swine gut or in the agriculture environment. Regardless of the mechanisms of selection, the results show that antibiotic use increased the abundance of resistance genes specific to and beyond the administered antibiotics from a diverse pool of background resistance genes in the swine microbiome, and that this increase was detectable even above a high background of resistance-gene diversity.

The collateral effects of antibiotics extend beyond influencing resistance genes. Statistical analysis of COGs in the swine metagenomes showed that genes encoding virulence, gene-transfer, and energy production and conversion functions are selected by in-feed antibiotics. Specifically overrepresented COGs included some relating to P pilus assembly; the P pilus has been described for attachment and virulence in *E. coli* (31). Additional COGs of interest in the medicated metagenome included transposases, which are known to participate in the transfer of antibiotic resistance genes (32). These functions could enhance the stability and spread of resistance genes in microbial communities. Additionally, an increase in the abundance of genes encoding energy production and conversion functions could be a factor in growth-promoting properties of at least some antibiotics, but further

experiments are required to test this. Antibiotics are thought to improve feed efficiency in agricultural animals primarily by decreasing the bacterial load, which is beneficial to the host by reducing competition for nutrients and decreasing the host's cost of responding to the microbes (2). Analysis of the swine metabolome after antibiotic treatment showed an effect on various biosynthetic pathways, including sugar, fatty acid, bile acid, and steroid hormone synthesis (33). COGs may therefore be useful signposts for identifying microbes and functions important to the performance-enhancing effects of antibiotics like ASP250.

Changes in microbial functions result from changes in microbial membership, and interesting membership shifts were detected. The decrease in Bacteroidetes in the treated animals may relate to the growth-promoting benefits obtained from feeding swine ASP250 as part of their diets. Obese mice have lower levels of Bacteroidetes relative to Firmicutes in their feces compared with lean mice (34). The obese mice have improved energy-harvesting capacity, presumably because of this shift, and perhaps this shift is related to improved feed conversion in swine. In addition, an increase in *E. coli* prevalence in response to oral antibiotic treatment has been reported for amoxicillin, metronidazole, and bismuth (35), metronidazole (36), and vancomycin and imipenem (37) in the mammalian gut microbiota. However, amoxicillin plus the β-lactamase inhibitor clavulanic acid administered both in the feed and intramuscularly resulted in decreased *E. coli* in pigs (38), and oral ciprofloxacin yielded decreased Proteobacteria populations in humans during treatment (39). These results are an important reminder of the varying collateral effects of different antibiotics. *E. coli* are both commensal and pathogenic inhabitants of mammalian gastrointestinal tracts; an increase in *E. coli* could be beneficial or harmful, either to the host or to the food chain. Additionally, increased *E. coli* populations associated with excessive weight gain in pregnant women (40)

is an unfavorable result in this host but parallels a potential growth-promoting role for this bacterium in livestock. The cost and benefit of a given antibiotic for a desired outcome must therefore be carefully weighed.

Differences among the rarer members of the microbial communities between treatment and control animals are less understood and invite further investigation. Of those that increased with treatment, members of the Deinococcus-Thermus phylum are known for being resistant to environmental stress; these organisms have only recently been identified in the human gut (41). In addition, Ruminococcus spp. are common in ruminants and are frequently found in the hindgut of pigs (42). Adept at degrading cellulose, an increase in Ruminococcus spp. after antibiotic treatment may aid in feed conversion in swine. Taken together, the data suggest numerous possibilities for how the swine gut microbiota might be involved with the improved feed efficiency afforded by certain in-feed antibiotics.

Conclusions

The results show that even a low, short-term dose of in-feed antibiotics increases the abundance and diversity of antibiotic resistance genes, including resistance to antibiotics not administered, and increases the abundance of *E. coli*, a potential human pathogen. Additionally, analysis of the metagenomes implicated functions potentially involved with improved feed efficiency. The study design featured environmental control in a single uniform inoculum source (the mother), control of the host genetics, no exposure of the sow or piglets to antibiotics except for the treatment, and identical diet except for the inclusion of ASP250 in one group. Future studies should include other in-feed antibiotics, multiple litters of swine with robust replication, and the identification of the antibiotic-induced mechanisms

that lead to increased feed efficiency. Implications of antibiotic resistance on human and animal health need to be taken into account when discussing agricultural management policies and evaluating alternatives to traditional antibiotics. With the use of antibiotics in animal agriculture at a crossroads, studies like this and others that highlight the collateral effects of antibiotic use are needed.

Materials and Methods

Swine.

Six pigs (siblings) were used in this study and were split into two groups of three: a group to receive antibiotics and a group to receive no antibiotics. Animals were raised in accordance with National Animal Disease Center Animal Care and Use Committee guidelines. The rooms housing the pigs were decontaminated before the beginning of the study. A pregnant sow was obtained from a hog farm at which she had no prior exposure to antibiotics. The piglets shared a pen with the sow for 3 wk after birth; her feces were therefore the primary bacterial inocula for the piglets. After weaning, all pigs were fed the same diet (TechStart 17–25; Kent Feeds) until the start of the study, at which point the medicated pigs were moved to a new clean room and given the above diet but containing ASP250 (chlortetracycline 100 g/ton, sulfamethazine 100 g/ton, penicillin 50 g/ton). Freshly voided feces were collected from nonmedicated and medicated animals just before treatment (medicated and nonmedicated day 0) and 3, 14 and 21 d after treatment.

DNA Sequencing.

Fecal DNA was isolated by bead-beating, and the V3 region of the 16S rRNA gene was amplified and sequenced. PCR products were sequenced on a 454 Genome Sequencer FLX, using the manufacturer's protocol for FLX chemistry (Roche Diagnostics). For sequencing the metagenome, DNA from the feces was pooled by treatment group (nonmedicated, medicated) for each time point (day 0, day 14). Day 14 samples were sequenced using FLX chemistry and day 0 samples were sequenced using Titanium chemistry (Roche Diagnostics).

Phylotype Analysis.

Only sequences longer than 50 bp were used for phylotype analysis (phylotyping), which totaled 133,294 sequences (70,667 unique sequences) from 12 fecal samples. After binning the samples by barcode, phylogenetic analysis and taxonomic assignments of the V3 portion of the 16S rRNA gene were made using the Ribosomal Database project Web tools (43). Additional phylotype comparisons and hypothesis testing were performed with the software package mothur (44). Bray-Curtis similarity coefficients were calculated from 16S rRNA gene sequence data from individual animals at 0 and 14 d and plotted in an NMDS graph to show the similarity among samples. MDS plots and analysis of similarities statistical tests were done in PAST (45).

Metagenomic Analysis.

Sequences were dereplicated and analyzed by BLAST against the nonredundant database and ARDB (27). The BLAST reports were parsed to extract COG information, and

COG frequencies were analyzed in ShotgunFunctionalizeR (46). The ARDB was kindly provided by Liu and Pop (27) so that we could perform BLASTx analyses locally. In both analyses, differences with $P < 0.05$ were significant, and the significant COGs were labeled with their respective COG category to visualize trends. For ecological analyses, the number of hits was normalized to 100,000 submitted reads and analyzed using NMDS and cluster analyses with the Bray-Curtis similarity measurement in PAST (45).

Quantitative PCR.

Primer sets were grouped into 18 resistance types by subjecting all primer sets to the ARDB BLAST tool (Supplementary table A4) or by the BLAST tool in the National Center for Biotechnology Information when no results were obtained by the ARDB BLAST (Supplementary table A5). Quantitative PCR primers, reagents, and DNA samples were loaded into six subarrays of OpenArray plates (Applied Biosystems) (47). For each 33 nL qPCR reaction, 1 ng of extracted DNA was added as template. Quantitative PCR reagents and conditions were preformed as previously described (47). Relative gene copy numbers were calculated as follows: gene copy number = $10(26-Ct)/(10/3)$, where Ct equals the threshold cycle (Supplementary table A6). Amplification curves were manually inspected using quality control measures. The abundance of the 16S rRNA gene was determined (48), and *E. coli* was quantified by using a *uidA* primer set (49). Copy numbers of the *uidA* and 16S rRNA genes were calculated in relation to a standard curve, which was generated by using 10-fold dilutions of 10^8 to 10^0 copies as template, in triplicate reactions. Those reactions targeting 16S rRNA and *uidA* were performed separately from the OpenArray platform.

Statistical Analysis of qPCR Results: Abundance and Diversity.

All qPCR data were normalized between samples by dividing the gene copy number by 16S rRNA copy number and subsequently natural log-transformed to achieve normal distribution. A repeated-measures ANOVA model was used to determine if treatment or time was significantly related to the abundance of antibiotic resistance genes and Shannon diversity in different samples. The best covariance structure of the residuals for each response variable was determined and used for repeated measures ANOVA testing (SAS v9.2; SAS Institute). A Bonferroni adjustment was not used in the comparison of resistance genes or resistance gene types because of excessive reduction in power of tests; therefore, the reported P values were not corrected for multiple comparisons.

Shannon diversity was calculated using PAST ver. 1.87 (45) using data normalized between samples (resistance gene copy number/16S rRNA gene copy number). Bray-Curtis coefficients were calculated for each of the samples using the natural log-transformed data (50). A two-way ANOSIM was calculated using these data, considering treatment and time as the two factors. Two-way ANOSIM analysis and NMDS plots were completed using the Bray-Curtis measure for β-diversity.

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Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. SRP004660).

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Figure 2.1. Shifts in fecal bacterial community membership with antibiotic treatment. (*A*) NMDS analysis of Bray-Curtis similarity coefficients calculated from 16S rRNA gene sequence data from individual animals at days 0 and 14 shows the similarity among replicate pig fecal samples. (*B*) Phylum-level composition of fecal microbial communities. Data were pooled for a given treatment and time point and are shown as percentage of abundance. (*C*) Genus-level composition of *Proteobacteria*, shown as the total number of sequences (normalized to 50,000 total reads). (*D*) Predicted genera of COG3188 homologs found in the swine metagenomes based on BLASTx analysis. COG3188 was overrepresented in the medicated metagenome vs. the nonmedicated metagenomes.

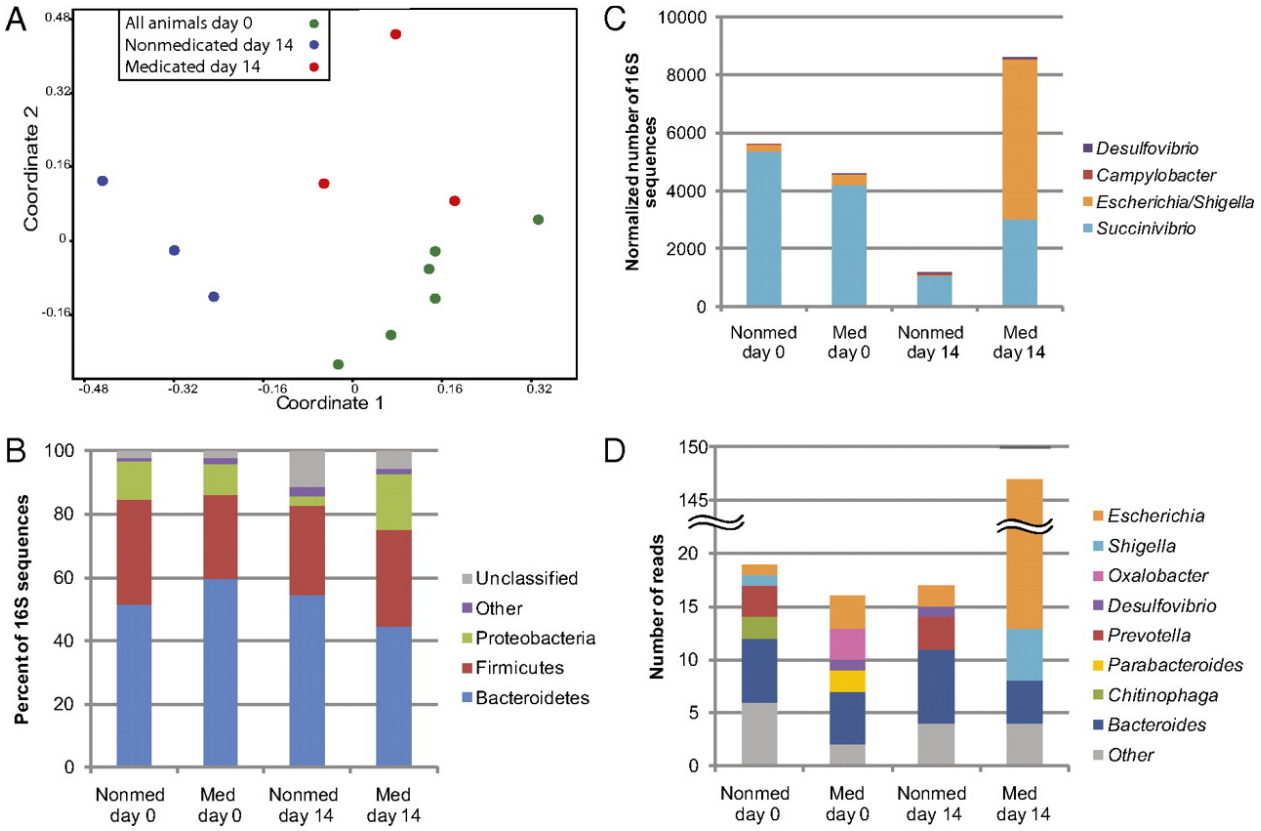

Figure 2.2. Changes in diversity and abundance of antibiotic resistance genes (ARG) in swine feces with antibiotic treatment. (*A*) Metagenomes were analyzed by BLASTx against the ARDB, and the number of reads were normalized to 100,000 total reads per metagenome. (*B*) Differences in the abundance of resistance genes were assessed by calculating the ratio of resistance gene copy number (ARG) to 16S rRNA gene copy number per sample as detected by qPCR. Columns denoted by the same letter are not statistically significant ($P > 0.05$) within each resistance type. Error bars represent the SEM. (*C*) Bray-Curtis similarity coefficients were calculated from qPCR-derived resistance gene abundance data and plotted in a multidimensional scaling graph. The distance between points indicates the degree of difference in the diversity of resistance genes between samples. The medicated sample outlier (square) is from one medicated pig on day 21. Measures for day 0 samples are not shown.

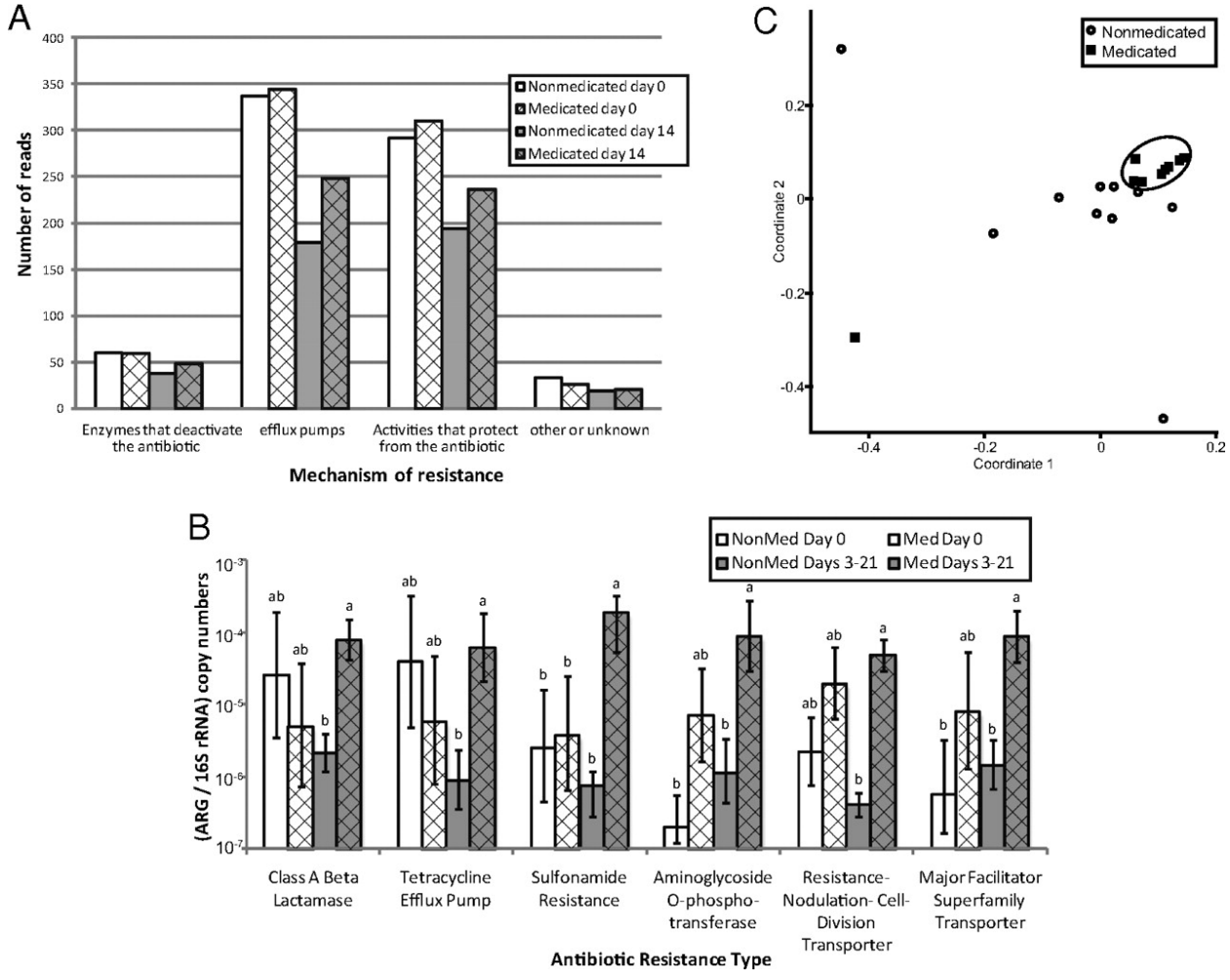

Table 2.1. Antibiotic resistance genes differentially represented ($P < 0.05$) in the medicated vs. nonmedicated pig fecal samples as detected by metagenomics [number of sequences in the medicated ($n = 1$) vs. nonmedicated ($n = 3$) metagenomes per resistance gene] and qPCR (gene copy number/16S rRNA gene copy number) during the treatment period

Mechanism of resistance	Gene(s) detected by: Metagenomics	Gene(s) detected by: qPCR	Confers resistance to
More prevalent in the treated metagenome			
ABC transporter system. Macrolide-lincosamide-streptogramin B efflux pump.	*lmrA*		Lincomycin
Aminoglycoside O-phosphotransferase. Modifies aminoglycosides by phosphorylation.	*aph(3'')-Ib*, *aph(6')-Ic*, *aph(6')-Id*	*aph(3'')-Ib*	Streptomycin
Class A β-lactamase. Cleaves the β-lactam ring.		bla_{TEM-1}, bla_{SHV-2}	β-Lactams
Major facilitator superfamily transporter, tetracycline efflux pump. Multidrug resistance efflux pump.	*emrD*, *mdfA*, *mdtH*, *mdtL*, *rosA*, *tet*(B)	*tet*(B), *bcr*	Chloramphenicol, tetracycline, deoxycholate, fosfomycin, Florfenicol, sulfathiazole
Resistance-nodulation-cell division transporter system. Multidrug resistance efflux pump.	*adeA*, *amrB*, *mdtF*, *mdtN*, *mdtO*, *mdtP*, *oprA*, *tolC*	*acrA*	Fluoramphenicol, aminoglycoside, macrolide, acriflavine, doxorubicin, erythromycin, puromycin, β-lactams
Ribosomal protection protein. Protects ribosome from inhibition by tetracycline.	*tet*(M)	*tet*(O)	Tetracycline
Sulfonamide-resistant dihydropteroate synthase. Cannot be inhibited by sulfonamide.	*sul2*	*sul2*	Sulfonamide
More prevalent in the control metagenomes			
Resistance-nodulation-cell division transporter system. Multidrug resistance efflux pump.	*mexF*		Chloramphenicol, fluoroquinolone
Ribosomal protection protein. Protects ribosome from inhibition by tetracycline.	*tetB*(P), *tet*(Q)		Tetracycline

CHAPTER 3. SWINE MICROBIAL COMMUNITIES SUBDIVIDED BY INTESTINAL LOCATIONS AND ANTIBIOTICS

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I designed this experiment with the listed coauthors. Additionally, I performed the necropsies, collected and processed all samples, contributed to the DNA extractions, performed all PCR reactions, analyzed the 16S rRNA data, analyzed part of the metagenomic data, and wrote this manuscript.

Abstract

Bacteria residing in the mammalian intestinal tract benefit from nutrient-rich feed and host-provided nutrients while assisting the host with digestion. Along the mucosa, bacteria are in close contact with the host and are important in the modulation of host health. Antibiotics are used to treat disease, prevent disease, and improve feed efficiency in swine, and have unknown effects on the spatial structure of bacterial communities on the mucosa and along intestinal tract. In this study, bacterial communities from specific gut locations were characterized to evaluate the effects of in-feed antibiotics on the bacterial spatial and functional diversity in the swine intestinal tract. Three-month-old swine were fed a diet containing performance-enhancing antibiotics (chlortetracycline, sulfamethazine, and penicillin [known as ASP250]), and after necropsies, tissue and lumen samples were collected from intestinal locations (ileum, cecum and colon) and feces. We used phylogenetic and metagenomic approaches to evaluate the spatial distribution of and the impact of antibiotics on the swine gut microbiota. Operational taxonomic unit (OTU) analysis revealed dramatic differences related to gut location and divergence of antibiotic-fed animals from those from control animals. While the ileum contents had minimal diversity (dominated by *Firmicutes*), the mucosa and other intestinal locations harbored diverse communities of microbes. A subset of organisms was found only on the tissue samples; these may be mucosal specialists. Antibiotic exposure lead to increased abundance of some antibiotic resistance genes, many which occur on plasmids, and genes associated with bacterial metabolism. Additionally, pigs receiving in-feed antibiotics had lower levels of many genera, but some did increased, including *Escherichia* and *Erysipelothrix*, both potential human and

animal pathogens. The results from this study demonstrate localized adaptations of microbial communities and function, and underscore the collateral effects of in-feed antibiotics.

Introduction

The mammalian gastrointestinal tract is a heterogeneous ecosystem with distinct, stratified environments. Along the length of the intestinal tract, resident bacteria benefit from nutrient-rich digesta, assisting the host in starch digestion *and vitamin synthesis (Bird et al 2010). Factors such as nutrient availability, pH, redox potential, and* peristalsis *strongly influence the composition of bacterial communities in the intestinal tract (Hao and Lee 2004). Along the* host mucosa, a subpopulation of bacteria thrive on host-provided nutrients, such as mucin and dead epithelial cells (Ashida et al 2012). Some of these bacteria are attached to the tissue while others burrow into the mucus layer. These mucosa-associated bacterial communities have been shown to differ from those in the intestinal contents (Zoetendal et al 2002), and those differences vary across the intestinal tract (Hill et al 2010). Because bacteria along the mucosa are in close contact with the host, it is important to define these bacteria, their functions, and their products. Key microbial features could be important in the modulation of host health, specifically regarding changes associated with diet, such as antibiotics or alternative “growth promotants”.

The spatial distribution of intestinal bacteria can be thought of as existing along two axes, a longitudinal axis (proximal to distal) and a radial axis (lumen to the mucosa) (Takahashi and Sakaguchi 2006), both of which serve to differentiate the nutrient and bacterial distribution. The human colon may harbor as many as 1,000 bacterial species, with 10^{11} cells per gram of feces (Whitman et al 1998), whereas studies of the small intestine

commensal bacterial showed much lower diversity (species richness and abundance) and cell densities (Booijink et al 2010). Previous studies looking at the radial organization in the mouse colon showed an enrichment of *Firmicutes* spp. on the mucosa, and an enrichment of *Bacteroidaceae, Enterococcaceae* and *Lactobacillaceae* spp. in the lumen (Nava et al 2011). In pigs with necrotizing entercolitis, more *Clostridium* spp. were associated with the ileal mucosa than in healthy pigs (Azcarate-Peril et al 2011). Observations like these illustrate the need to include spatial distribution when describing bacteria in the gut to better understand their niches and how they impact host health and performance.

Antibiotics are used in livestock to treat disease, prevent disease, and improve feed efficiency (Cromwell 2002, Dibner and Richards 2005). Studies looking at shifts in microbial communities with in-feed antibiotics have detected shifts in bacterial membership and functions in swine feces (Allen et al 2011, Looft et al 2012), but don't shed light on localized communities in the gut. While bacterial numbers are highest in the feces, they may not be representative of the upstream intestinal tract (Nielsen et al 2003). Feces often serve as a proxy for the gut microbiota because they can be harvested readily and frequently, but fecal bacteria are a mix of potentially discrete upstream populations. Direct sampling of specific gut locations allows for differentiation of sub-communities of the gut microbiota, potentially revealing how performance-enhancing antibiotics affect bacterial taxa in these micro-habitats. Microscopic changes in the bacteria along the ileal, cecal, and colonic epithelium have been shown in chicks receiving in-feed antibiotics (Chichlowski et al 2007). Understanding how in-feed antibiotics affect the membership and functional distribution of mucosal bacteria may inform alternatives to antibiotics that mimic these changes.

In this study we examined the longitudinal and radial distribution of bacterial communities in the swine intestine. Within both axes there exists many micro-habitats and niches both in the lumen and mucosa, but for comparisons we present them here as distinct, singular communities. To this end, we characterized the bacterial membership and functions at three gut locations using phylotype and metagenomic analyses. Additionally, the effect of the in-feed antibiotic ASP250 was evaluated. The results show dramatic differences in the longitudinal and radial compositions of bacterial communities in the swine intestinal tract. The mucosa-associated communities were diverse, with as much species richness or greater than that found in the lumen. Pigs receiving in-feed antibiotics had higher levels of particular gut bacteria, including some that were specific to the mucosa. Additionally, antibiotic exposure lead to increased abundance of genes that confer resistance to certain antibiotics and of genes associated with bacterial metabolism.

Methods

Swine.

Piglets were acquired and managed as previously described (Allen et al 2011). Briefly, piglets were divided into two groups of six at approximately three-months-old, with equal representation of littermates and gender. All pigs were fed the same diet (TechStart® 17-25, Kent Feeds, Muscatine, IA) until the start of the experiment, at which point six control pigs continued to receive TechStart while the other group received TechStart containing ASP250 (chlortetracycline 100 g/ton, sulfamethazine 100 g/ton, penicillin 50 g/ton) *ad libitum* until euthanization.

Between 15 and 20 weeks of age, each animal was euthanized by intracardiac injection of sodium pentobarbital (26% solution containing 1 ml/4.5 kg Sleepaway; Fort Dodge Laboratories, Overland Park, KS) after being anesthetized with an intramuscular injection of a cocktail of ketamine (8 mg/kg), xylazine (4 mg/kg), and Telazol (6 mg/kg, Fort Dodge Animal Health, Fort Dodge, IA), in accordance with National Animal Disease Center Animal Care and Use Committee guidelines. Three sections of the GI tract (the ileum, the cecum, and the mid-colon) were harvested immediately after euthanasia. The sections were tied off by string ligatures and placed on ice for transport to the lab. With each intestinal sample, a both a tissue and a lumen-content sample was taken for DNA extraction. Tissues were washed by gently flowing sterile phosphate-buffered saline (PBS) across the tissue until no contents were visible but attached bacteria remained. A sterile glass microscope slide was used to scrape any attached bacteria off of the surface for DNA extractions. These tissue scrapings plus corresponding gut contents and feces were collected from each pig, and DNAs were extracted using the manufacturer's protocol with the UltraClean Soil DNA Isolation Kit (Mo Bio Laboratories, Solana Beach, CA, USA).

16S rRNA gene sequence analysis.

Amplification of the V1-V3 region of bacterial 16S rRNA genes was carried out as previously described (Allen et al. 2011). Briefly, the conserved primers 8F (5′-AGAGTTTGATCCTGGCTCAG) (Weisburg et al 1991) and 518R (5′-ATTACCGCGGCTGCTGG) (Muyzer et al 1993) were designed with attached eight-nucleotide unique sequence barcodes (Allen et al 2011, Hamady et al 2008). PCRs were performed with 22 cycles, and products were separated by gel electrophoresis and purified

using the MinElute kit (Qiagen Inc., Valencia, CA). They were then sequenced on a 454 Genome Sequencer (GS) FLX using the manufacturer's protocol for Titanium chemistry (Roche Diagnostics, Branford, CT).

Phylotype analysis.

Sequence data were processed per Roche's protocols, followed by AmpliconNoise (Quince et al 2011), mothur (Schloss et al 2009, Schloss et al 2011), and Uchime (Edgar et al 2011) to remove barcodes and reduce sequence artifacts produced during PCR and sequencing. Uchime was implemented in mothur (Schloss et al 2009). OTU-based phylogenetic analysis (97% similarity cutoff) and hypothesis testing were performed with normalized data in mothur (Schloss et al 2009). To visualize changes in bacterial diversity, a nonmetric multidimensional scaling (NMDS) plot and dendogram of these data using the Bray-Curtis index of OTUs was calculated in PAST (Hammer et al 2001). The estimated total richness of operational taxonomic units was calculated by Catchall (Bunge 2011) within mothur. The bacterial richness estimate resulting from the best parametric model was reported for each sample. Taxonomic assignments of the 16S rRNA gene sequences were made using the Ribosomal Database project (RDP) web tools (Cole et al 2009). The Metastats statistical software was used for making comparisons between samples and identifying trends (White et al 2009). A p-value less than 0.05 with a q-value (false discovery rate) less than 0.05 was considered significant and *R* between 0-0.3 was considered a slight correlation while *R* greater than 0.3 was considered a correlation.

Metagenomic analysis.

DNAs from feces and gut contents (of the ileum, cecum, and midcolon) were sequenced on Roche's GS-FLX instrument (Roche Diagnostics) and processed as described previously (Allen et al 2011). Four samples from each of 12 pigs, minus samples from four pigs that were missing either ileal or fecal material, yielded 44 total DNA samples. These samples were divided across four regions of two Titanium plates. DNA sequences were de-replicated (Gomez-Alvarez et al 2009) and analyzed using the metagenomic and BLASTx pipelines in CAMERA (https://portal.camera.calit2.net, (Seshadri et al 2007)). Additionally, metagenomes were blasted in-house against the antibiotic resistance genes database (Liu and Pop 2009) as described previously (Looft et al 2012). Frequency counts of all functional data were normalized to the lowest total number of open reading frames (clusters of orthologous groups [COGs]), of assigned reads (SEED subsystems), or of bacterial reads (resistance genes) among the 44 metagenomes. Normalized datasets were tabulated and analyzed for patterns (e.g. cluster analyses and ANOSIM) in PAST (Hammer et al 2001). Additionally, STAMP (Parks and Beiko 2010) was used to make statistical comparisons between samples. Because the metagenomic samples might not be independent (e.g. the ileum contents move downstream to become the cecal contents), the Kruskal-Wallis H-test was used rather than ANOVA, followed by the Games-Howell post-hoc test and Storey's FDR multiple comparison correction (Parks and Beiko 2010). Finally, COGs were further scrutinized by the Poisson distribution using the po.stat function in ShotgunFunctionalizeR (Kristiansson et al 2009). To draw statistical conclusions, the following cut-offs indicated: $0.5 < P \leq 0.1$, trend; $P \leq 0.05$, significance; R=0-0.3, slight correlation; R=0.3-0.5, medium correlation.

Results and discussion

Swine intestinal bacterial diversity exhibits radial specificity.

The overall bacterial diversity included common intestinal bacterial groups, dominated by *Firmicutes* (35%), *Bacteroidetes* (21%), *Proteobacteria* (3%), and *Spirochaetes* (2%), as shown previously in pigs (Allen et al 2011, Lamendella et al 2011, Looft et al 2012). Bacterial communities with either tissue or lumen association were different. Of the differences that were statistically significant, only three genera were found at higher levels in the lumen (*Erysipelothrix, Anaerovorax, and Oxalobacter* [$p<0.01$]) than on the mucosa (Figure 3.1). *Erysipelothrix*, includes *E. rhusiopathiae,* the etiological agent of swine erysipelas and causes opportunistic infections in humans, both resulting in major economic costs (Shimoji 2000). It is noteworthy that most intestinal bacteria identified from the lumen were also found on the mucosa, suggesting that the ability to occupy the mucosa is shared by most gut microbes.

Although most lumen-associated microbes were also found on the mucosa, some notable organisms were found in greater abundance on the mucosa. Exclusive mucosa-associated bacteria included *Pseudomonas*, *Campylobacter*, *Bradyrhizobium*, *Weissella*, and *Sutterella* ($p<0.01$) (Figure 3.1). *Pseudomonas* spp., for example, are well adapted to colonize the mucosa, including by degrading mucin (Aristoteli and Willcox 2003, Tsang et al 1994). *Pseudomonas* spp. have been identified in human intestinal studies at low levels (Shinohara et al 2010), and colonize other mucosal surfaces in the human (Aristoteli and Willcox 2003). *Campylobacter* spp., which are common intestinal bacteria in the pig and common foodborne pathogens of humans, can effectively penetrate the mucus layer of the mucosa with its corkscrew motility to reach the epithelial layer (On 1996). The lactic acid

bacteria *Weissella* spp. and the bile-degrading *Sutterella* spp. have both been isolated from the human intestinal mucosa (Green et al 2006, Heilig et al 2002), suggesting that these genera are broadly specific to mucosal surfaces across mammals. Many of these mucosal colonizers are not dominant members of the gut community, but rather are part of a milieu of specialized intestinal bacteria that have adapted to colonize the mucosa. Studies that focus on fecal bacterial communities miss these subpopulations that were only captured by direct sampling of the mucosa.

Distinct radial differences in the ileum.

By additionally taking the gut location into account when examining radial diversity, the ileum in particular revealed striking differences between the bacterial communities of the lumen and mucosa. Only 15 OTUs (±5) were detected in the lumen compared to 252 OTUs (±115) associated with the host tissue at the same ileal location. This is likely in part because of both the low digesta retention time in the small intestine and the low bacterial diversity coming out of the stomach. The difference between the richness of the tissue and lumen communities at the other gut locations was not significant, and the luminal richness at these locations was higher than in the lumen of ileum (741 OTUs (±370)). Although the ileal lumen supported limited diversity, the bacterial richness of the mucosa reflected that seen in the other intestinal sections (Figure 3.2B).

The elevated richness (membership) on the ileal mucosa compared to the lumen, and the high richness found on the other mucosal sites, may be important for intestinal function and health. In most cases, the mucosal samples contained a similar collection of genera as the corresponding lumen section. The richness of the ileum mucosa, however, more closely

resembled the mucosa at other locations than the adjacent lumen. By providing a rich environment for bacteria, the mucosa may provide the bacterial inoculum needed to aid in the breakdown of non-digested food substrates (Williams et al 2011). Savage (1987) proposed that the mucosal bacterial populations in the gut both stabilize the intestinal community and prevent pathogens by providing a constant, established inoculum for the lumen to complete digestion. Our data show comparable richness on the mucosa and in the lumen, suggesting that Savage's hypothesis is worth further investigation. Further research is needed to evaluate the importance of mucosal colonization as it relates to the gut ecosystem, intestinal function, and host health.

Bacterial membership and functions exhibit longitudinal specificity.

The small intestine and colon have considerably different physiological functions, and their respective bacterial communities reflect these differences. Analysis of the longitudinal differences of the bacterial phyla within each sampled intestinal section shows phylum-level differences between the ileum and the cecum, colon, and feces (Figure 3.2a). Finer resolution of the longitudinal + radial differences shows genus-level differences across lumen and mucosa of the sampled intestinal locations (Figure 3.2b). Members of the *Synergistetes* phylum were identified in each intestinal section, and increased in each subsequent section; ileum < cecum ($P < 0.001$, q= 0.001), cecum < mid-colon ($P < 0.001$, q=0.024). *Synergistetes* have been identified in a diverse range of anaerobic environments, but have few cultured representatives (Vartoukian et al 2007). *Synergistetes* have previously been identified in the intestinal tract of mammals, but their role in the gut is not known (Marchandin et al 2010). Additionally, species-level analyses of operational taxonomic units

(OTUs) revealed significant differences related to gut location (P<0.01, R=0.65). The ileal contents (both control and medicated) showed reduced diversity (richness and abundance) when compared to the rest of the intestinal tract communities (Figure 3.3a). Differences were driven by the dominance of members of the *Firmicutes* phylum (*Clostridium* and *Turicibacter*) in the ileum and various genera in the colon (*Prevotella, Oscillibacter,* and *Succinivibrio*).

Analysis of the metagenomes further supported the longitudinal stratification of gut bacteria, showing the ileum metagenomes to be particularly different. Indeed, more ileal contents sequences were annotated as eukaryotic (37 % ±19%) than in any of the other locations (remaining samples were < 1%) (Supplementary figure B1), reinforcing the limited bacterial richness detected in the ileum by the 16S rRNA sequence analysis. When all of the various COG data were reduced into single datapoints per metagenome, the richness of COGs in the ileum separated from those of the other gut locations in a pattern consistent with the bacterial membership (Figure 3.3b, Figure 3.3c). Significant drivers of the functional differences included increased abundance of respiration and amino acid SEED subsystems in the ileum. Interestingly, the stress response subsystem increased in abundance from ileum to feces (R^2=0.31, Supplementary figure B2), suggesting that the gut environment becomes less hospitable for bacteria towards its distal end.

The effect of antibiotics on bacterial community structure and function.

Many changes were detected in the membership and functions of the bacterial communities across all intestinal locations with antibiotic treatment. Analysis of the community structure at the 0.03 OTU level indicated divergence of the antibiotic-fed swine microbiota from the non-medicated swine microbiota (P < 0.01, R=0.21) (Figure 3.4). The

phyla that changed after exposure to antibiotics were the *Spirochaetes*, *Synergistetes*, *Planctomycetes*, *Fibrobacteres*, *TM7*, and *Actinobacteria* decreasing with antibiotic treatment ($p<0.01$) and *Tenericutes* increasing with treatment ($p<0.01$). Many genera decrease with exposure to antibiotics, including *Treponema*, *Succinivibrio*, *Streptococcus*, *Parasporobacterium*, and *Anaerovorax* ($p<0.01$). Certain genera increased with antibiotic treatment, such as *Escherichia*, *Lachnobacterium*, *Tannerella*, *Anaeroplasma*, and *Erysipelothrix* spp. ($p<0.01$) (Figure 3.5). *Lachnobacterium* spp. increase in both the lumen and mucosa with antibiotics and is known to produce bacteriocins (antimicrobial compounds) and butyrate, the latter of which is an energy source for colonic epithelial cells. For this reason, *Lachnobacterium* spp. have been considered as a potential direct-fed microbial for use in livestock production (McAllister et al 2011).

Shifts in *E. coli* populations with ASP250 and other antibiotics have been previously shown (Allen et al 2011, Looft et al 2012). *Erysipelothrix* spp. isolated from swine have been identified harboring resistance genes, including tetracycline (Yamamoto et al 2001). *Tannerella* spp. have been associated with periodontal disease in humans and form biofilms in the presence of mucin, which may impart some resistance to antibiotics (Roy et al 2011).

Bacterial functions showed congruent separation by antibiotic treatment as was seen in phylotype analysis (Figure 3.3c). Among the COGs assigned to individual metagenomes, 112 were differentially expressed with antibiotic treatment (83 higher in the medicated and 29 higher in the non-medicated) (Figure 3.6). The COG categories C (energy production and conversion), G (carbohydrate transport and metabolism), M (cell wall/membrane/envelope biogenesis), P (inorganic ion transport and metabolism), and S (function unknown) were all overrepresented in the significant COGs of the medicated animals (Figure 3.6). The

prevalence of these COGs in the medicated animals suggests a more active microbiota, with more “tools” to exploit the available nutrients in the gut. Numerous studies have demonstrated the many host benefits of bacterial fermentation within the gut (Hooper et al 2002, Sunkara et al 2011). The COG category C genes are associated with in feed antibiotics in swine, and may indirectly benefit the host (Looft et al 2012). Categories G and M are both associated with transport and metabolism and point to increased microbial activity.

The effect of in-feed antibiotics is measurable at discrete gut regions.

Some antibiotic effects are location specific. After antibiotic treatment, *Helicobacter* spp. were reduced to non-detectable levels in the cecum ($p<0.01$) while *Holdemania* spp. increased in the cecum ($p<0.01$) and along the mucosa ($p<0.01$). *Helicobacter* spp. have been associated with livestock disease (Dewhirst et al 2000, Kirkbride et al 1985) and cecal colonization of laboratory animals (Fox and Lee 1997). The reduction in *Helicobacter* spp. in the intestine may benefit the host by reducing inflammation along the mucosa. *Holdemania* is related to *Erysipelothrix* according to 16S rRNA gene analysis (Willems et al 1997) and was shown to be enriched on the chicken cecal mucosa (Gong et al 2002). *Holdemania* spp. have a unique murein type as part of their cell wall structure, which may contribute to insensitivity to penicillin (one of the antibiotics in ASP250) (Willems et al 1997).

Antibiotic resistance gene diversity.

Diverse antibiotic resistance genes were detected in both ASP250 treated and non-treated animals, with 213 different resistance genes detected across all metagenomes. Among the antibiotic resistance genes found, 12 were higher in the medicated metagenomes and

eight were higher in the control metagenomes (Table 3.1) ($p<0.05$). Six genes were previously identified as increasing in abundance in a study of four metagenomes from swine receiving in-feed antibiotics (Looft et al 2012). Additional resistance genes that were higher in the medicated metagenomes included genes conferring resistance to chloramphenicol, cephalosporins, penicillins, and aminoglycosides. Penicillin antibiotics are part of the ASP 250 cocktail, and the direct selection for penicillin resistance genes is a likely outcome. Chloramphenicol and aminoglycoside resistance in *E. coli* has been shown to be co-selected in swine (Rosengren et al 2007). Previous studies have also found cephalosporin resistance in swine intestinal bacteria, including *E. coli* and *Salmonella* (Kijima-Tanaka et al 2003, Winokur et al 2000). Cephalosporin use in agriculture has recently received attention. The FDA has proposed rules limiting the "off label" use of cephalosporin drugs in animal agriculture because it is used in human health, and drug resistance is a concern.

Conclusions

These results highlight the spatial distribution of bacterial communities in the mucosa and lumen of the intestinal tract. The spatial distribution of bacterial membership and function points to the localized adaptations of commensal bacteria. Although adjacent in the gut, the microbiota of the ileum differs drastically from that of the cecum and colon. All mucosa locations harbored diverse bacterial members, including some genera that were not found in the lumen. Several genera specific to the mucosa shifted with ASP250 use, and their impact on host health deserves additional research. Collateral effects of in-feed antibiotics include the increased *Escherichia* and *Erysipelothrix* populations with ASP250, which are concerning because these species represent potential human or animal pathogens. COGs

associated with energy conversion and metabolite transport in the microbiota of medicated swine suggest that antibiotics select for members of the community that could be more metabolically active. A transcriptomic analysis of the fecal microbiota would be ideal to test this hypothesis. ASP250 also selected for antibiotic resistance genes, 19 of which were higher in the medicated metagenomes. Several of these are relevant to animal and human health and are subject to ongoing debates in Congress. The controversy over the use of antibiotics in agriculture is likely to continue, but understanding how growth-promoting antibiotics affect intestinal bacterial communities, both along the mucosa and in the lumen, may inform alternative strategies that preserve the performance benefits while reducing potential risks to human and animal health.

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Figure 3.1. Bacterial genera with significantly different representation between mucosa and lumen environments across all intestinal locations, based on taxonomic inference of bacteria (16S rRNA sequences) (P<0.01; q<0.05).

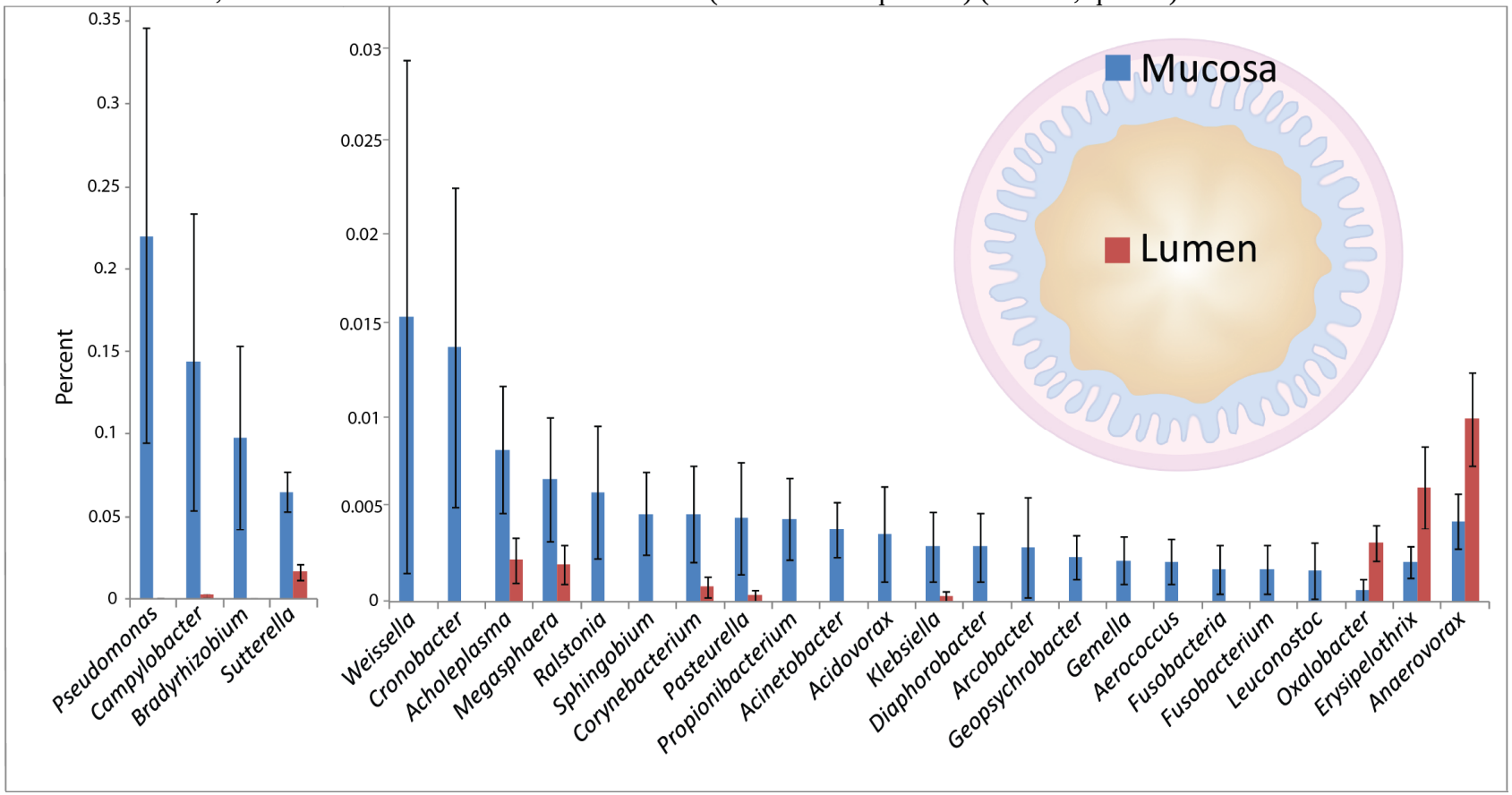
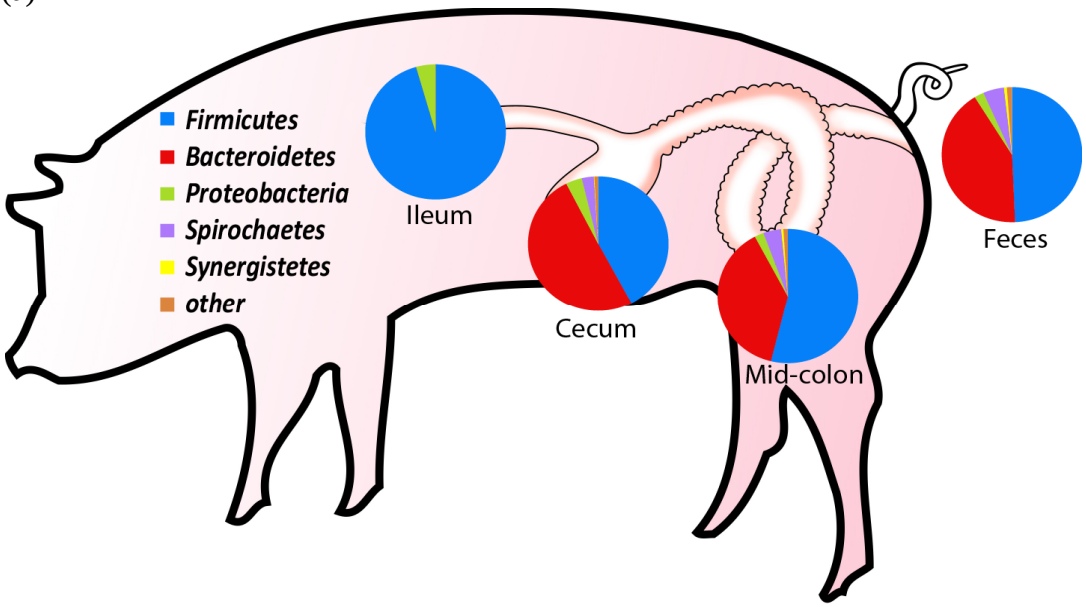


Figure 3.2. Swine intestinal bacterial communities differentiate along longitudinal and radial axes. (a) Phylum-level distribution of luminal bacterial through the intestinal tract; (b) Spatial distribution of bacteria in the swine intestinal tract, based on taxonomic inference of bacteria (16S rRNA sequences). Tissue samples=T, Lumen contents=C.

(a)



(b)

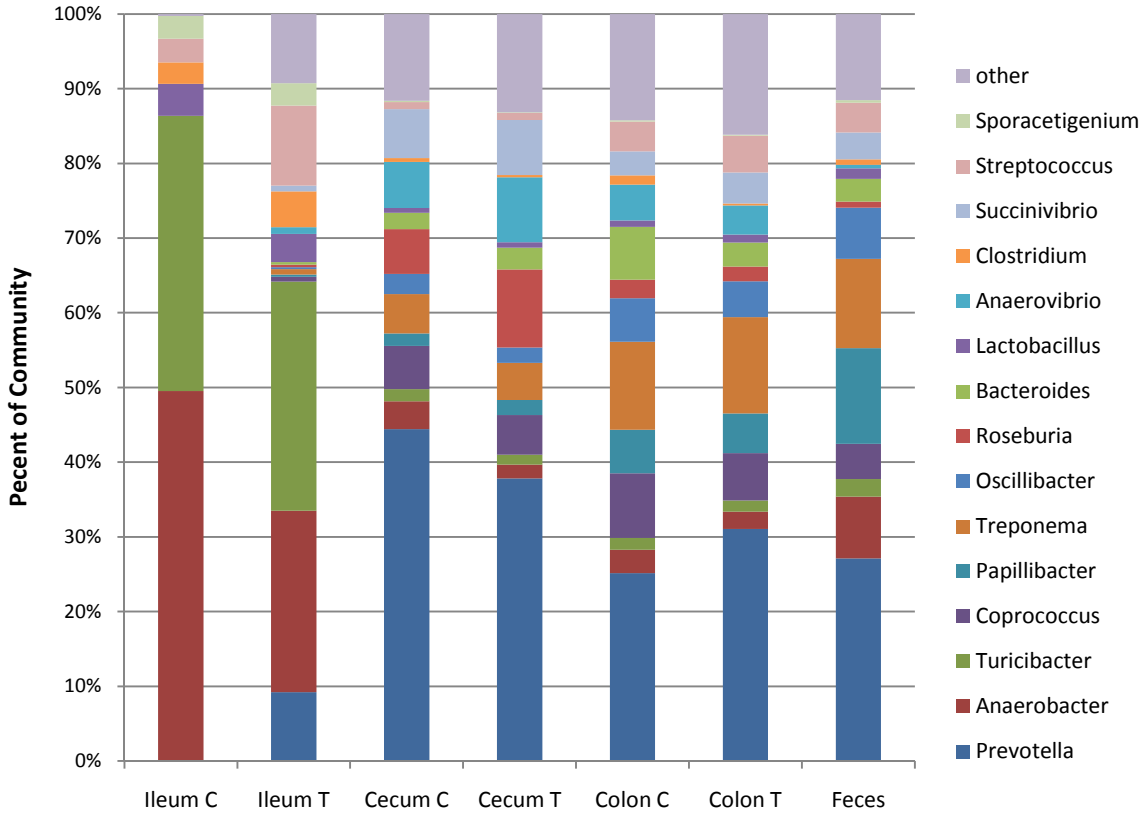


Figure 3.3. Bacterial community structure. (a) Heatmap of normalized OTU data (0.03 similarity cutoff) from medicated (med) and non-medicated (non-med) communities averaged by treatment and location. Each column in the heatmap represents a different OTU, and the color of the OTU for each sample is scaled between black (low-abundance) and red (high-abundance) according to the relative abundance of that OTU within the group. (b) Cluster analysis of OTU data (bray-curtis similarity measure), averaged by treatment and location. (c) Cluster analysis of COG assignments from metagenomic data (dice similarity measure), averaged by treatment and location. L = lumen, M = mucosa, red = medicated, black = nonmedicated

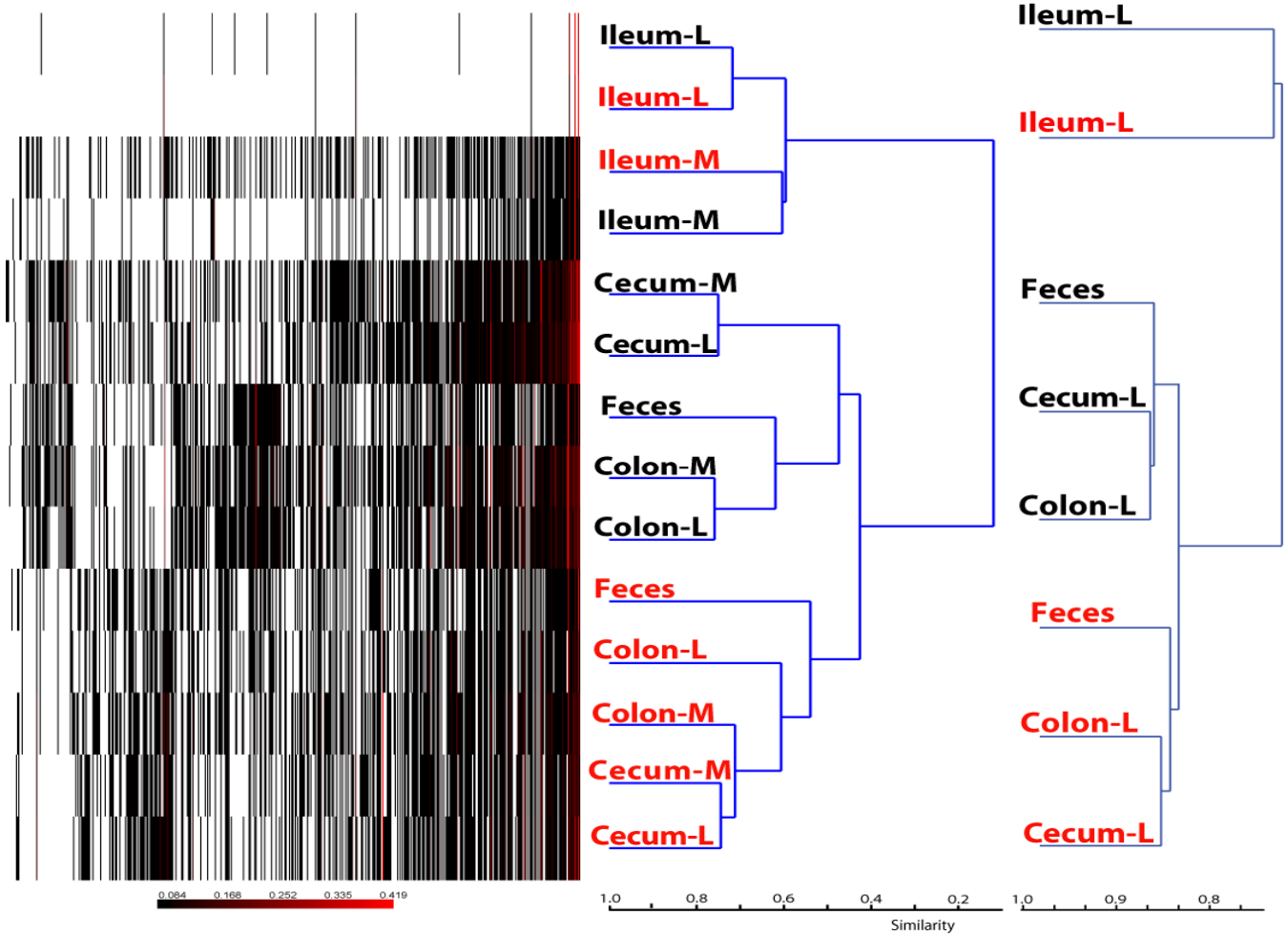


Figure 3.4. Nonmetric multidimensional scaling analysis of OTU-based bacterial 16S rRNA gene sequence abundances in individual pig intestinal samples. Environmental variables are plotted as vectors. The length of each vector is arbitrarily scaled, so only their directions and relative lengths should be considered. Mucosal and lumen samples are not differentiated. Ileum= diamond, cecum= triangle, mid colon= square, and feces= circle.

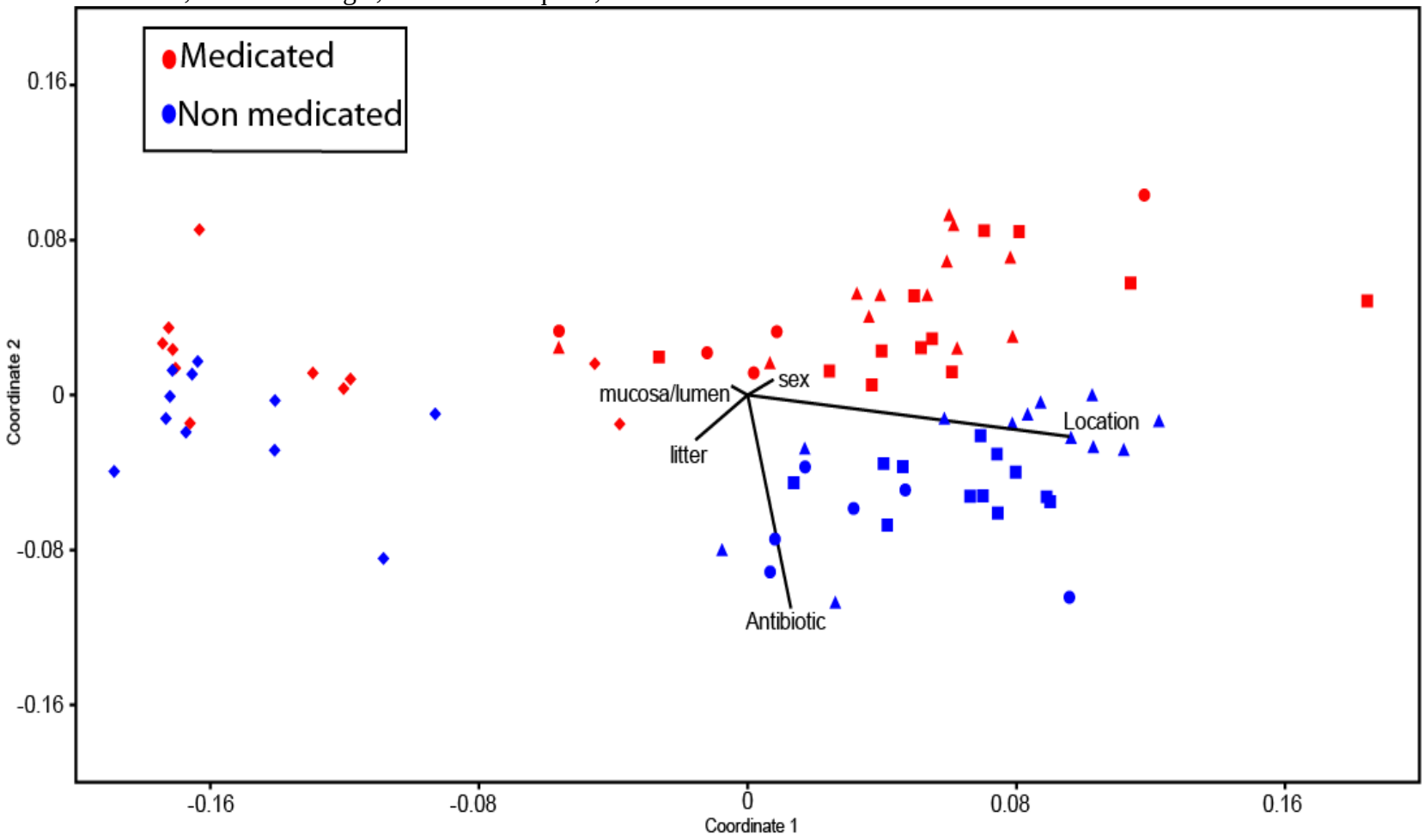


Figure 3.5. Bacterial genera that are differentially present due to antibiotic treatment, based on taxonomic inference of bacteria (16S rRNA sequences) from intestinal locations (P<0.01, q<0.05). Data are pooled across intestinal locations.

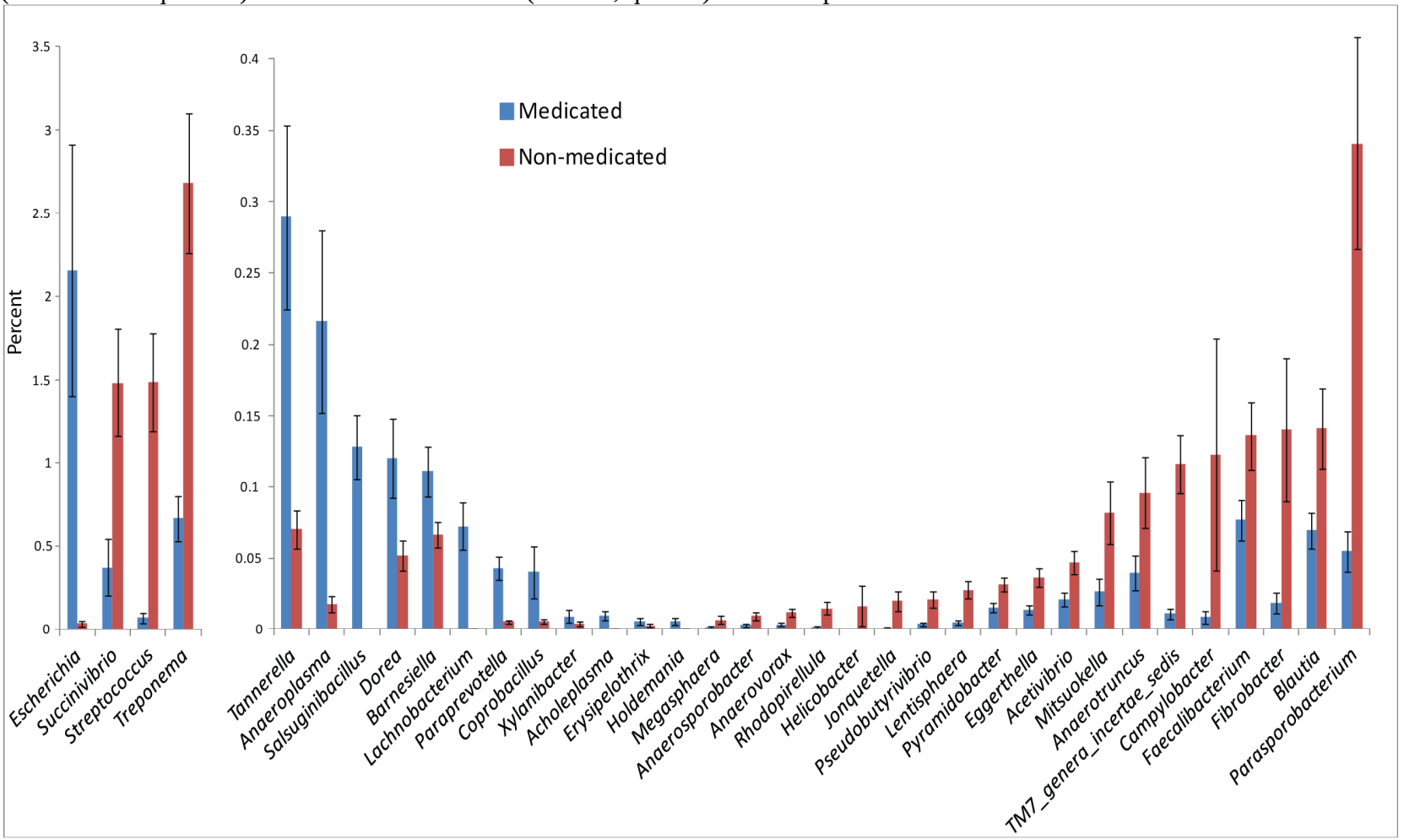


Figure 3.6. Spindle diagram of COGs with differential representation (adjusted $P<0.01$) between the medicated and nonmedicated metagenomes, grouped by higher level COG category. COG categories more frequently significant in the medicated metagenomes are highlighted in gray.

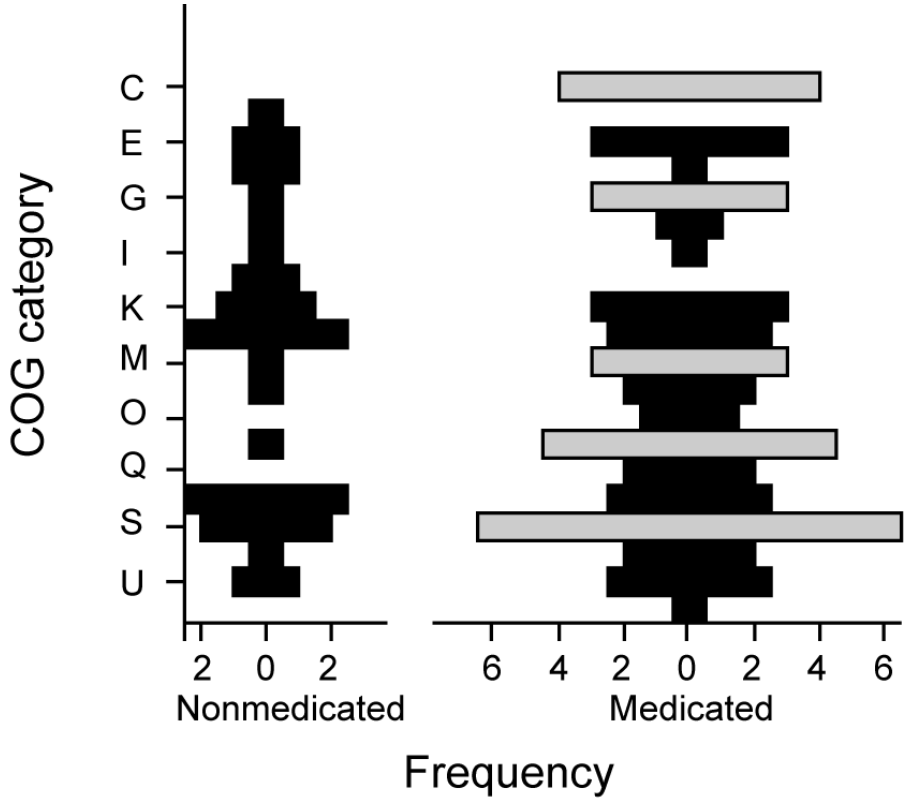


Table 3.1. Antibiotic resistance of differential abundance in swine medicated versus nonmedicated metagenomes.

Mechanism of resistance	Genes detected	Confers resistance to	Found in genus[a,b]	Reported on mobile elements?
Increased in medicated metagenomes				
Multidrug resistance efflux pumps	*cmlA*	chloramphenicol	*Acinetobacter, Aeromonas, Enterobacter, Escherichia, Klebsiella, Proteobacteria, Pseudomonas, Salmonella, Serratia, Staphylococcus*	yes
	emrE	aminoglycoside	*Escherichia, Shigella*	no
	mdtH	deoxycholate, fosfomycin	*Citrobacter, Cronobacter, Enterobacter, Escherichia, Klebsiella, Salmonella, Shigella*	no
	mdtL	chloramphenicol	*Citrobacter, Escherichia, Salmonella, Shigella*	no
	mdtM	acriflavin, chloramphenicol, norfloxacin	*Citrobacter, Escherichia, Salmonella, Shigella*	no
	mdtO, mdtP	acriflavine, puromycin	*Escherichia, Salmonella, Shigella*	no
	tet(B)	tetracycline	*Actinobacillus, Escherichia, Haemophilus, Neisseria, Pasteurella, Pseudomonas, Salmonella, Serratia, Shigella, Streptococcus*	yes
Enzymes that deactivate the antibiotic	*aph(3'')-Ib*	streptomycin	*Acinetobacter, Actinobacillus, Aeromonas, Bordetella, Esherichia, Haemophilus, Klebsiella, Listeria, Pasteurella, Pseudomonas, Salmonella, Shigella*	yes
	bla_{ampC}	cephalosporins	*Escherichia, Shigella*	yes
Activities that protect from the antibiotic	*arnA*	polymixin	*Citrobacter, Esherichia, Salmonella, Shigella*	no
	$vanW_B$	vancomycin	*Clostridium, Enterococcus*	yes
Decreased in medicated metagenomes				
Multidrug resistance efflux pumps	*macB*	macrolide	*Citrobacter, Enterobacter, Escherichia, Klebsiella, Salmonella, Shigella*	no
	msrA	lincosamide, macrolide, streptogramin B	*Arcanobacterium, Corynebacterium, Enterococcus, Gemella, Pseudomonas, Staphylococcus, Streptococcus*	no
	tet(L)	tetracycline	*Actinobacillus, Bacillus, Bifidobacterium, Enterococcus, Lactobacillus, Paenibacillus, Pseudomonas, Staphylococcus, Stenotrophomonas, Streptococcus*	yes
Activities that protect from the antibiotic	*ermB*	erythromycin	*Bacillus, Bacteroides, Clostridium, Enterococcus, Escherichia, Lactobacillus, Lactococcus, Neisseria, Staphylococcus, Streptococcus*	yes
	ksgA	kasugamycin	*Citrobacter, Cronobacter, Enterobacter, Escherichia, Klebsiella, Pectobacterium, Salmonella, Serratia, Shigella, Yersinia*	no
	tet(M)	tetracycline	*Aeromonas, Clostridium, Enterococcus, Escherichia, Klebsiella, Lactobacillus, Lactococcus, Listeria, Pseudomonas, Streptococcus*	yes
	$vanY_A$	vancomycin	*Bacillus, Enterococcus, Paenibacillus, Staphylococcus*	yes
	$vanY_B$	vancomycin	*Clostridium, Eggerthella, Enterococcus*	yes

[a]According to the ARDB
[b]Up to 10 genera are listed

CHAPTER 4. *CLOACIBACILLUS PORCORUM* SP. NOV.,–A MUCIN-DEGRADING BACTERIUM FROM THE SWINE INTESTINAL TRACT

Looft T., Levine U. Y., Stanton T.B.

I isolated CL-84^T as part of a survey of mucin degraders in the swine gut. I also performed all experiments described in this manuscript, with the following exceptions: cellular fatty acid compositions were determined by Microbial ID Inc. (Newark, DE), Judy Stasko helped performed the electron microscopy work, and Uri Levine helped performed the butyryl-CoA:acetate CoA-transferase assay.

Abstract:

A novel anaerobic, mesophilic, amino-acid-fermenting bacterium, designated strain CL-84^T, was isolated from the swine intestinal tract on mucin-based media. The bacterium had curved-rod cells (0.8-1.2 µm x 3.5-5.0 µm), stained Gram negative, and was non-motile with no evidence of spores. CL-84^T produces acetate, propionate, formate and butyrate as the end products of metabolism when grown on serine. Optimum growth occurred at 39°C and pH 6.5. The major cellular fatty acids were iso-$C_{15:0}$, iso-$C_{15:0}$ 3-OH, iso-$C_{17:0}$, and $C_{16:0}$, distinguishing CL-84^T from closely related species. The G+C content of the DNA was 55.1 mol%. 16S rRNA gene sequence analysis showed that CL-84^T had sequence similarity with characterized genera within the Phylum *Synergistetes*, Family *Synergistaceae.* Phylogenetic analysis showed that CL-84^T was related, but distinct from *Cloacibacillus evryensis.* Based on these findings, we propose that strain CL-84^T represents a new species of the genus *Cloacibacillus*. We further propose the name *Cloacibacillus porcorum* be designated for this species. The type strain is CL-84^T (=DSM, =ATCC).

Introduction

Members of the recently described *Synergistetes* phylum have been identified in a diverse range of anaerobic environments, including anaerobic digesters (Ganesan *et al.*, 2008) and some infections in the human body (e.g. peritoneal fluid, soft tissues, blood and periodontal pockets) (Vartoukian *et al.*, 2007). Despite *Synergistetes'* culture-independent identification in a wide range of environments, there are few cultured representatives of this phylum. *Synergistes jonesii*, the first characterized *Synergistetes* species, was isolated from a goat rumen. *S. jonesii* degrades toxic pyridinediols in the animals' diet, and in turn the

animal's gut provides required nutrients (Allison *et al.*, 1992). In this paper we describe the isolation of a mucin-degrading bacterium, strain CL-84^T, from the swine intestine and describe a new species of *Cloacibacillus*. To our knowledge, this is the first description of a member of the Phylum *Synergistetes* that utilizes mucin as its sole source of carbon.

Strain isolation.

Strain CL-84^T was one of eight *Synergistetes* strains isolated during the characterization of mucosa-associated and mucin-degrading microorganisms from the swine intestinal tract. The gently rinsed mucosal surface of a pig cecum was scraped with a sterile microscope slide and inoculated into minimal medium containing mucin. A series of three enrichments (10 days each) in broth, containing a basal medium, described below, and 1% wt/vol hog gastric mucin (HGM) (Sigma-Aldrich Corp., St Louis, MO), were used to improve the recovery of mucolytic bacteria before inoculation on solid media. Mucin-degrading bacteria were isolated on solid basal medium supplemented with 1% wt/vol HGM after incubation at 39°C for five days. Pure cultures were obtained after isolates were streaked for isolation three times. All cultures were inoculated and incubated (39°C) in a Coy anaerobic chamber inflated with an atmosphere of N_2 (85%), CO_2 (5%), and H_2 (10%). The basal medium contained (per liter) 0.45 g of $CaCl_2$, 0.45 g of $MgSO_4$, 2.25 g of KH_2PO_4, 2.25 g of K_2HPO_4, 4.5 g of NaCl, 4.5 g of $(NH_4)_2SO_4$, 0.05% cysteine, 0.5 g hemin^{-1}, 0.0001% resazurin, and 1.6% noble agar.

Strain CL-84^T cell morphology.

Isolate CL-84^T grew optimally on brain-heart infusion broth with 0.05% cysteine, 0.0001% resazurin, and supplemented with 20 mM arginine and histidine (BHIAH). This medium was used to maintain cultures. After three days of growth at 39°C on BHIAH medium, CL-84^T reached a terminal OD_{620}, of 1.2, representing 1.5 x 10^9 CFU/ml. The calculated doubling time was 8 hrs. Isolate CL-84^T cells cultured in BHIAH broth had a curved-rod shape, were non-motile, and spores were not seen (Figure 4.1a). On BHIAH plates after 5 days of growth CL-84^T produced small semi-translucent brown colonies that were 1 mm in diameter. Gram strain was negative. Ultrathin sections were prepared for transmission electron microcopy (TEM) from four-day-old cultures, stained with uranyl acetate and lead citrate, and examined with a Tecnai 12 G^2 Biotwin microscope (FEI, Hillsboro, OR). TEM micrographs showed a cell outer envelope structure consistent with gram negative cells with a thin peptidoglycan layer surrounded by an outer membrane (Figure 4.1b). No spores or inclusion bodies were seen. CL-84^T was variable in size and ranged from 0.8-1.2 µm X 3.5-5.0 µm. The TEM appearance and Gram staining characteristics are consistent with the Gram negative envelope characteristics of other *Synergistetes spp.*

16S rRNA gene sequence analysis.

Genomic DNA was extracted from a cell pellet of the isolate following Dale and Greenway (1985). PCR amplification was carried out as described previously (Downes *et al.*, 2005), with the conserved bacterial primers 8F (Wilmotte *et al.*, 1993) and 1492R (Stackebrandt & Goodfellow, 1991). Purified PCR products were sequenced yielding nearly full-length (1447 bp) sequences for the CL-84^T 16S rRNA gene. Taxonomic assessments of

the 16S rRNA gene sequences were made using the Ribosomal Database project (RDP) web tools (http://rdp.cme.msu.edu/) (Cole *et al.*, 2009), which placed CL-84^T within the *Synergistetes* phylum. The closest type strain matches to *Cloacibacillus evryensis* (95% sequence identity) and *Synergistes jonesii* (90% sequence identity). *C. evryensis* was isolated from a municipal anaerobic waste digester (Ganesan *et al.*, 2008), and *S. jonesii* was isolated from a goat rumen (Allison *et al.*, 1992). Support for other *Synergistetes* bacteria being associated with mucus and possibly utilizing mucin can be found from their identification from subgingival plaque samples, and optimized growth (in co-culture) by the addition of mucin to the media (Vartoukian *et al.*, 2010).

Neighbor-joining, maximum parsimony, and maximum likelihood phylogenetic analysis was performed based on the alignment of the 16S rRNA gene sequence from CL-84 with Silva's SINA web aligner (Pruesse *et al.*, 2007) in the software package ARB (Ludwig *et al.*, 2004). The methods used to construct the tree were ARB neighbor-joining (10,000 bootstrap replicates), maximum parsimony with DNAPars v3.6 (1,000 bootstraps), and maximum likelihood with RAxML ('advanced bootstrap + refinement of BS tree' algorithm, GTRGAMMA model, 1,000 bootstraps). All three analyses produced trees with the same topology, and therefore only the neighbor-joining tree is presented (Figure 4.2). Isolate CL-84^T grouped with uncharacterized isolates from infected human blood (accession numbers GQ258969, EF551162, EF551160) (Marchandin *et al.*, 2010) and peritoneal fluid samples (DQ412721) (Horz *et al.*, 2006). These sequences were approximately 99% similar to each other, to CL-84^T, and form a cluster previously designated OTU cluster 2 by Ganesan *et al.* (2008). In contrast, the 16S rRNA gene sequences from *C. evryensis* and *S. jonesii* were only 95% and 90% similar to the sequences in OTU cluster 2, respectively. CL-84^T, and related

sequences, form a distinct branch from *C. evryensis* (OTU cluster 1), within the *Cloacibacillus* genus. A previous study of the swine gut microbiota also identified a sequence from an uncultured *Synergistetes sp.* (AF371930) in the pig gut, which is distantly related to CL-84^T and groups with the *Pyramidobacter* genus (Figure 4.2) (Leser *et al.*, 2002).

As part of a larger survey of swine intestinal microbes, the genome of isolate CL-84^T was sequenced using 454 titanium pyrosequencing platform (454 Life Sciences, Branford, CT). Preliminary sequence analysis revealed CL-84^T to have a G+C content of 55.1 mol%.

Substrate utilization.

Mucins are glycoproteins with carbohydrate side chains connected to a protein backbone by O-glycosidic links (Nataro, 2005) and are the major component of mucus. The carbohydrate side groups are made of the sugars galactose, fucose, N-acetylgalactosamine, N-acetylglucosamine, sialic acid, and mannose (Allen *et al.*, 1998). Mucolytic bacteria use proteases and glycosidases to degrade host mucin at polypeptide and glycosidic bonds, respectively (Bradshaw *et al.*, 1994). Growth on different mucin components was examined by preparing basal medium containing 0.2% w/v yeast extract with 0.5% w/v of each one of the following: chondroitin sulfate sodium salt, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, hyaluronan biotin sodium salt, mannose, N-acetylneuraminic acid, D-galactose, or fucose. CL-84^T was further evaluated for its ability to degrade mucin O-linked glycans, purified from the HGM, as previously described (Martens *et al.*, 2008). Growth on a variety of amino acids was examined by supplementing basal medium containing 0.2% w/v yeast extract with 10 mM of each one of the following: arginine, histidine, lysine, serine,

tryptophan, alanine, glutamate, aspartate, proline, glycine, cysteine, phenylalanine, isoleucine, leucine, valine, threonine, methionine, glutamine, asparagine, or tyrosine. Growth results are summarized in Table 4.1 as OD_{620} values.

Fermentation Products.

The supernatants from cultures on each substrate were analyzed for fermentation acids by gas chromatography of butyl esters (Salanitro & Muirhead, 1975; Stanton & Lebo, 1988). Major fermentation products were formate, acetate, or propionate; however, growth on serine, both alone and with threonine and proline, also produced buyrate (Table 4.1). CL-84^T contains butyryl-CoA:acetate CoA-transferase (E.C. 2.8.3.8) activity (Spec Act = 9.88±0.45 µmol min^{-1} mg protein^{-1}) as determined using cells cultured on basal medium with serine. The assay for transferase activity was performed on French press extracts of CL-84^T according to Buckel et al. (1981) except that it was evaluated at 412 nm, 39°C, with 0.1 mM butyryl-CoA, and 0.5 units (8.8 nkat) of citrate synthase. The most prevalent amino acids in mucin are serine and threonine (Schrager, 1970) CL-84^T's ability to produce butyrate when grown on serine may reflect its role in the gut, as butyrate has been shown to stimulate mucin synthesis in human colonic cell lines and animal mucosa (Finnie *et al.*, 1995; Hatayama *et al.*, 2007).

Additional biochemical characterizations were performed using Rapid ID 32 A and API ZYM test strips (bioMérieux, l'Etoile, France). CL-84^T showed positive arginine dihydrolase and glutamic acid decarboxylase activities and weak alkaline phosphatase and proline arylamidase activities. Additionally, the API ZYM test showed that CL-84^T had acid

phosphatase, naphthol-as-bi-phosphohydrolase, alkaline phosphatase, esterase (C 4), esterase lipase (C 8), and leucine arylamidase activities. CL-84^T tested negative for catalase.

Growth Tolerance.

Heat, pH, and oxygen tolerance were tested in BHIAH medium (see above). The pH range for growth of CL-84^T was between pH 4-8, with optimal growth (reaching OD_{620} >1.0) between pH 6-7. Growth was observed between 20ºC-45ºC. No viable cells remained after heating cultures to 80ºC for 30 min. Growth of CL-84^T was inhibited by NaCl concentrations above 1.4% w/v, with optimal growth at 0.6-0.8% w/v. CL-84^T cells were aerotolerant and still viable after 24hrs of exposure to oxygen. Growth of CL-84^T was not observed on BHIAH plates, exposed to 1.0 % oxygen, after one week of incubation.

Cellular fatty acid content.

The closest related type strain to CL-84^T, *C. evryensis*, was obtained from the DSM culture collection (No.19522, 158^T). Both CL-84^T and *C. evryensis* were grown in BHIAH medium, and cell pellets were used for cellular fatty acid analysis. The cellular fatty acid composition was determined by Microbial ID Inc. (Newark, DE) with the GC-based MIDI Sherlock® Microbial Identification System. The major cellular fatty acids for CL-84^T were iso-$C_{15:0}$ (27.1%), iso-$C_{15:0}$ 3-OH (15.0%), iso-$C_{17:0}$ (11.7%), and $C_{16:0}$ (9.7%). This profile differed from that of *C. evryensis* in both the fatty acid types and the proportions of each (Table 4.2).

Resistance to antibiotics.

Antibiotic minimum inhibitory concentrations (MIC) assays were modified from Allen *et al.* (2009) by incubating anaerobically in BHIAH for one week. CL-84^T was susceptible to tylosin, lincomycin, chlortetracycline, penicillin, florphenicol, ceftiofur and carbadox (MIC < 4 µg/µl) and resistant to kanamycin, vancomycin, and sulfathiazole (MIC is 512 µg/ µl each). Kanamycin (aminoglycoside) and sulfa-drug antibiotics are commonly added to swine feed for performance and disease prevention (Apley *et al.*, 2012; Kobland *et al.*, 1984). Genes encoding resistance for these antibiotics have been identified in swine metagenomes (Looft *et al.*, 2012). Other *Synergistetes* spp. have also been shown to be resistant to vancomycin (Allison *et al.*, 1992; Downes *et al.*, 2009; Ganesan *et al.*, 2008).

Description of *Cloacibacillus porcorum* sp. nov.

porcorum (por.co′rum. L. n. *porcus* swine, pig; L. masc. pl. n. *porcorum* of/from pigs).

Cells are obligately anaerobic, non-motile, with a curved-rod shape. Cells ferment amino acids, some mucin sugars, and produce acetate, propionate, and formate but only produced butyrate when serine was supplied, as the end products of metabolism. Cells have Gram-negative cell wall structure and range in size from 0.8-1.2 µm wide to 3.5-5.0 µm long. Strong growth is obtained in brain-heart infusion medium, and growth is enhanced by the addition of histidine, arginine, but not glucose. After 7 days incubation, colonies are 1 mm in diameter, circular, shiny, brown, and semi-translucent. The main cellular fatty acids are: iso-$C_{15:0}$, iso-$C_{15:0}$ 3-OH, iso-$C_{17:0}$, and $C_{16:0}$. Cells are resistant to kanamycin, vancomycin, and sulfathiazole and are susceptible to tylosin, lincomycin, chlortetracycline, penicillin, florphenicol, ceftiofur and carbadox. Optimal growth is at 39°C at pH 6.5.

The type strain, strain CL-84^T (=DSM, =ATCC), was isolated from the mucosal lining of a pig cecum in Ames, Iowa, USA. The DNA G+C content of the type strain is 55.1 mol%.

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Figure 4.1. (A) Phase contrast micrograph of CL-84^{T} cells grown in BHIAH medium. (B) Transmission electron micrographs of ultra-thin sections of CL-84^{T}, showing a loose outer cell membrane consistent a Gram-negative outer cell envelope.

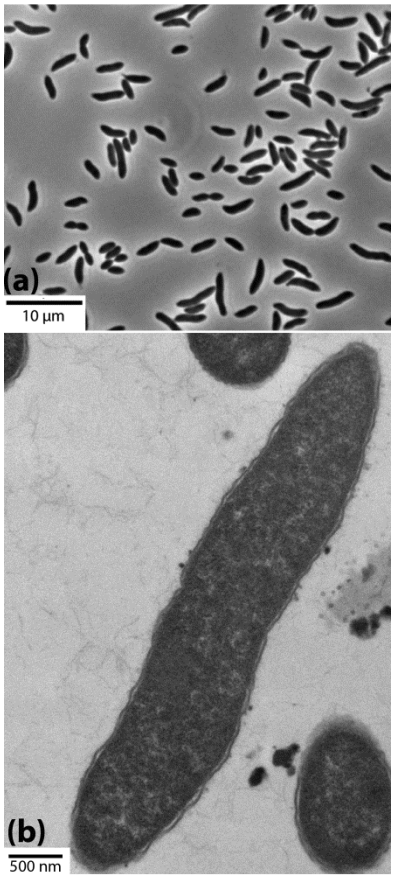

Figure 4.2. Neighbor-joining phyogentic tree of proposed species *Cloacibacillus porcorum* CL-84^T and selected reference sequences' partial 16S rRNA genes (all sequences ≥1334 bp). The tree is rooted by *Aminiphilus circumscriptus* strain, numbers by the branches of the tree represent the percentage bootstrap values of 10,000 resamplings, and are only noted if the percentage was greater than 50%. The scale bar represents 10 nucleotide substitutions per 100 nucleotides.

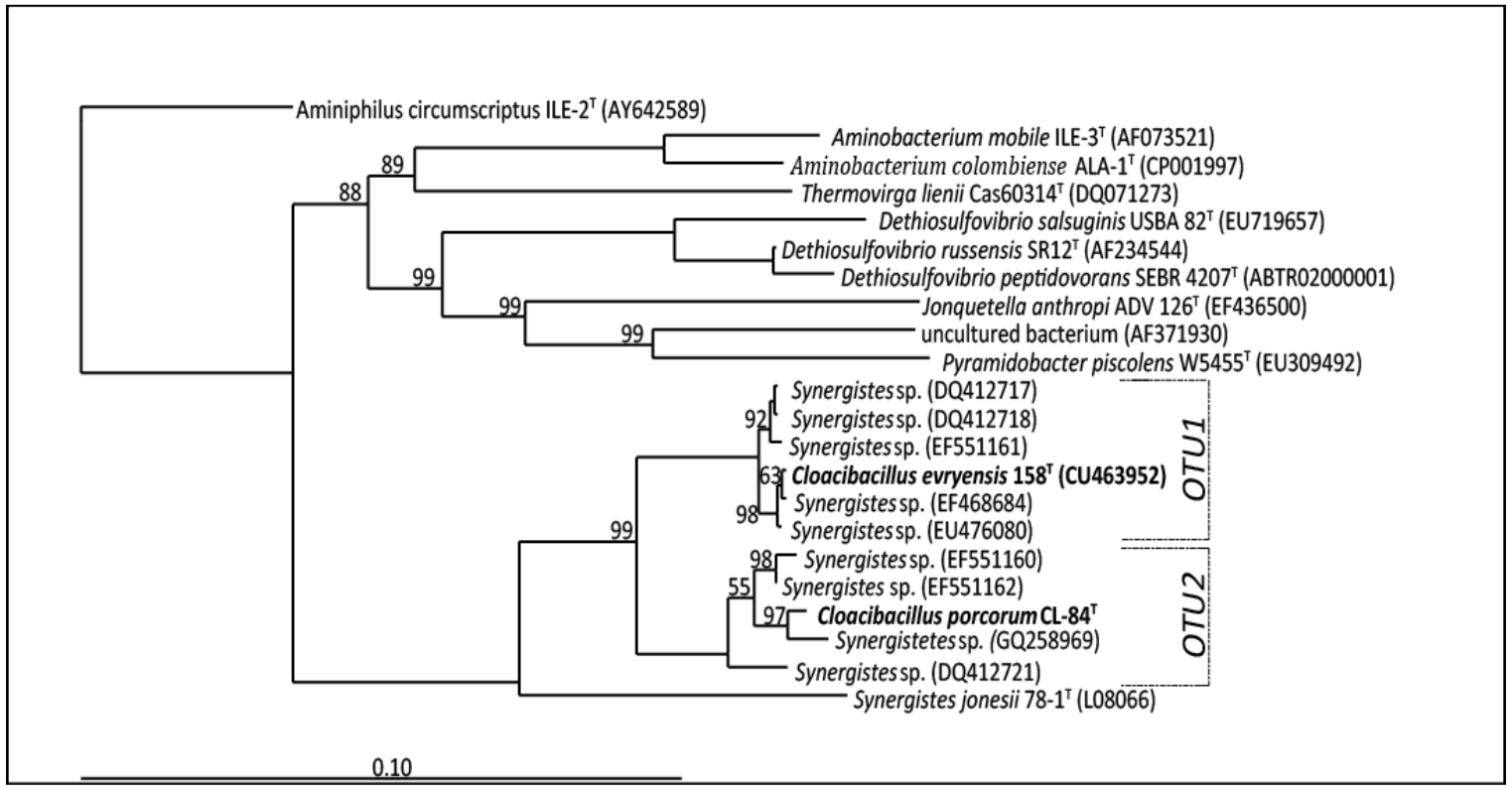

Table 4.1. Fermentation products from substrates that support growth of strain CL-84^T in basal meduim.

Substrate	Formic acid*	Acetic acid*	Propionic acid*	Butyric acid*	OD_{620}‡
BHIAH	-	++	+	-	+++
Histidine	+	+	+	-	++
Arginine	+	+	+	-	++
Tryptophan	-	+	-	-	+
Cysteine	+	++	+	-	++
Threonine	-	+	-	-	+
Serine	+	+	-	+	+
Serine, threonine, proline	+	++	+	+	++
(-)-N-acetylneuraminic acid	+	-	+	-	+
Hyaluronan biotin sodium salt	+	+	+	-	++
Mannose	+	+	-	-	++
Chondroitin sulfate sodium salt	+	+	+	-	+
Fucose	-	+	+	-	+
D-Galactose	-	+	-	-	+
N-acetyl-D-galactosamine	-	-	+	-	+
N-acetyl-D-glucosamine	-	-	-	-	+
Mucin	+	+	+	-	+
Mucin O-linked glycans	+	++	+	-	+

*Measure by gas chromatography. All values were corrected for the small amount of short chain fatty acids formed in the control tubes. Experiments were conducted in triplicate and mean values are given. + =1.0-5.0 mM, ++ =5.1-10.0 mM

†No growth was observed on the following substrates: Methionine, Aspartate, Valine, Proline, Isoleucine, Lysine, Alanine, Phenylalanine, Leucine, Glucose, Glutamine, Arginine, Glycine, Asparagine, Glutamate, and Tyrosine

‡ OD_{620} + =0.05-0.1
++ =0.2-0.5
+++ =0.6-1.2

Table 4.2. Cellular fatty acid profiles (%) of strain CL-84^T and *C. evryensis*, grown on BHIAH medium.

	% of total fatty acids	
	CL-84^T	***C. evryensis***
iso-$C_{15:0}$	27.1	14.2
iso-$C_{15:0}$ 3-OH	15	14
iso-$C_{17:0}$	11.7	2.4
$C_{16:0}$	9.7	1.8
iso-$C_{13:0}$	7.6	5.8
$C_{17:0}$	4.2	12
iso-$C_{17:1}$ at 10	3.9	2.7
$C_{16:1}$ *w7c*	3.7	1.6
$C_{18:0}$	2.8	0
$C_{18:1}$ *w11c*	2.6	0
$C_{17:1}$ *w11c*	2.3	12.4
$C_{15:0}$	1.9	4.8
$C_{16:1}$ *w11c*	1.7	0
iso-$C_{17:1}$ at 9	1.6	0
$C_{13:0}$	0	3.4
$C_{15:0}$ 3-OH	0	7.7
$C_{15:1}$ *w6c*	0	4.3
$C_{16:1}$ *w5c*	0	1.8
$C_{17:1}$ *w3c*	0	1.3
$C_{17:1}$ *w6c*	0	5.3
Summed Feature 2*	3.2	2.2
Summed Feature 6*	0	1.8

*Summed features represent two fatty acids that cannot be separated with the MIDI system.

Table 4.3. Differential characteristics of the species *Cloacibacillus porcorum* and related species in the phylum *Synergistetes*

Characteristic	**Cloacibacillus porcorum strain CL-84^T**	**Cloacibacillus evryensis strain 158^T***	**Synergistes jonesii Strain 78-1^T†**
Habitat	Swine intestinal tract	Anaerobic digester	Goat rumen
Cell morphology	Slightly curved rods	Straight rods	Rods
size	0.8-1.2 x 3.5-5.0 µm	0.8 - 1.0 x 2.0 - 3.0 µm	0.6-0.8 x 1.2-1.8 µm
16S rRNA similarity to CL-84^T (%)	100	95	90
Optimum growth temperature (°C)	39	35–40	39
DNA G+C content (mol%)	55.1	55.8	58
Metabolism	Fermentative	Fermentative	Fermentative
Fermentation	Proteolytic, some carbohydrates	Proteolytic	Proteolytic
Short chain fatty acids produced during fermentation	Acetate, propionate, and formate. Butyrate, only when serine is supplied	acetate, propionate, butyrate, and valerate	Acetate and propionate. Formate only when histidine is supplied
Major cellular fatty acids	iso-$C_{15:0}$, iso-$C_{15:0}$ 3-OH, iso-$C_{17:0}$, and $C_{16:0}$	iso-$C_{15:0}$, iso-$C_{15:0}$ 3-OH, $C_{17:1}$ *ω11c*, $C_{17:0}$	$C_{17:0}$, C_{20} cyc, $C_{17:1}$*ω6c, $C_{15:0}$*
Additional features	Degrades mucin	Some species of this genus detected in human clinical infections	Degrades 3,4-dihydroxy pyridine

***(Ganesan *et al.*, 2008)**
†(Allison *et al.*, 1992)

CHAPTER 5. GENERAL CONCLUSIONS

Collateral impacts of in-feed antibiotics on the swine microbiota

The data in this dissertation describe the shifts in swine intestinal bacterial communities with in-feed antibiotics using phylotype- and functional-based approaches. The design of these studies featured a single inoculum source (the mother), no exposure of the sow or piglets to antibiotics except for the treatment, and identical diets except for the inclusion of ASP250 for the medicated animals. Used for disease prevention, disease treatment, and growth promotion, ASP250 (chlortetracycline, sulfamethazine, and penicillin) caused significant community-wide changes in bacterial membership and functions.

Community Shifts

Many bacterial taxa decreased with antibiotic treatment, but a few increased. The shift in *E. coli* populations was particularly striking, increasing 10-fold with antibiotic treatment. *E coli* has been previously reported as increasing with other antibiotics such as amoxicillin, metronidazole, and bismuth (Antonopoulos et al 2009), metronidazole (Pelissier et al 2010), and vancomycin and imipenem (Manichanh et al 2010). While *E. coli* is a known intestinal commensal, it is also a potential human pathogen. Increasing *E. coli* numbers so dramatically with antibiotic exposure could be troublesome in the event that a pathogenic *E. coli* are part of this population.

Other notable shifts in microbial membership were detected. Members of the *Bacteroidetes* phylum decreased in the treated animals, which may relate to the growth-promoting benefits obtained from feeding ASP250 as part of their diets. Obese mice have

lower levels of *Bacteroidetes* relative to *Firmicutes* in their feces compared with lean mice, and this balance is associated with improved energy-harvesting capacity (Ley et al 2006, Turnbaugh et al 2006). In-fact, analysis of the metagenomes showed that bacterial functions associated to energy production and conversion were increased in the antibiotic-fed pigs. Our data suggest that the gut microbiota relates to the improved feed conversion obtained with in-feed antibiotics. Further studies are needed to confirm our results and define the mechanisms involved.

Resistance

Antibiotic resistance genes were pervasive in the swine intestinal microbiome, even in the absence of selective pressure. Antibiotic resistance genes were identified by metagenomic and qPCR analyses, and all genetically encoded mechanisms of resistance were detected: efflux pumps, antibiotic-modifying enzymes, and modified or protected targets of the antibiotic. The constant selective pressure of decades of in-feed antibiotics has lead to a high background level of resistance in the swine microbiome that persists even without antibiotics.

Even low, short-term exposure to in-feed antibiotics increased the abundance and diversity of antibiotic resistance genes, despite the high background. Some resistance genes associated with antibiotics not administered increased in abundance with in-feed ASP250. Many of the other antibiotic resistance genes matched genes that are known to occur on plasmids. This suggests an indirect mechanism of selection, perhaps by co-occurrence on mobile genetic elements conferring resistance to ASP250 antibiotics. The clustering of resistance genes on mobile elements, such as plasmids, may offer a fitness advantage to a

bacterium living in the presence of antibiotics. Antibiotic resistance genes occurring on plasmids is concerning because of the potential for transfer to pathogens, pointing to another undesirable collateral effect of in-feed antibiotics.

The importance of spatial distribution of bacteria in the gut

The longitudinal and radial distribution of bacterial communities in the swine intestine reflects interesting differences along both axes. The most dramatic differences were seen between the ileum and the rest of the intestinal tract. Bacterial communities from contents from the ileum were dominated by only a handful of species from the *Firmicutes* phylum, while the cecum and colon had several hundred across many phyla. Dramatic differences were also seen between the ileal lumen and mucosa, the latter of which had 10-fold more species than the former. A small subset of organisms was found only on the tissue, indicating potential mucosal specialists. While feces is easy to obtain and is a suitable material to characterize intestinal microbes for many studies, the spatial distribution of the microbes is lost when looking at feces alone. Defining the habitat in the gut provide insights on how microbes contribute to digestion in each intestinal section, on which microbes are in direct contact with the host, and on how the bacteria relate back to animal health.

Characterizing bacterial specialists in gut

The functional niche of mucin degradation was used to selectively culture bacteria that were adapted to a physical GI niche (mucosal colonization). Mucin-degrading activities have been linked to pathogenesis in some microbes (Campieri and Gionchetti 2001), but it may simply reflect an adaptation for mucosal colonization by commensal, beneficial

microbes. I described isolate *Cloacibacillus porcorum*, a new species of the *Synergistetes* phylum that was isolated from a pig cecal mucosa on mucin-based media. *Synergistetes* species have been identified in a diverse range of anaerobic environments (Vartoukian et al 2007), including in the culture-independent and -dependent studies in this dissertation. As a member of an understudied phylum, the significance of *Cloacibacillus porcorum* in the gut in unclear, but its *in vitro* phenotypes of mucin degradation and butyrate production suggest that it benefits from and provides benefit to host tissue.

Future directions

Much is still unknown about the role of intestinal bacteria in host health and how antibiotics affect the interaction between bacterial communities and their animal host. In order to develop viable alternatives to antibiotics, we need to know how the in-feed antibiotics are functioning to improve feed efficiency. In-feed ASP250 is only one of many antibiotics used for disease prevention and growth performance. Studies of the effects on the microbiota with other in-feed antibiotics are needed to identify common changes to the bacterial community with improved growth performance. Quantitative PCR should be used to confirm shifts identified with sequencing-based studies.

Community-wide transcription analysis (metatranscriptomics) may provide insights over metagenomic studies because it examines the activity of the community, not just the metabolic potential. This would be useful in identifying the mechanisms bacterial communities use to deal with the stress of antibiotics, and how energy-harvesting functions benefit the host. Additionally, analysis of host gene expression along the intestinal tract will

allow us to detect host responses to bacteria along the mucosa, thus defining the mechanisms of the mutualism between a host and its microbiota.

Bacteria that interact with the host are important, and the data in this dissertation suggest that there is much more to the story. In particular, it was surprising to find much higher diversity on the ileal mucosa relative to the ileal lumen. The colon is often the focus of gastrointestinal microbial studies, but many intestinal infections happen in the small intestine, which is an underexplored ecosystem. Studies characterizing bacterial communities further up the intestinal tract (small intestine through stomach) will help define their role in animal health.

While culture-independent studies have greatly expanded our understanding of GI bacterial communities, having isolates in pure cultures is invaluable for defining their characteristics and relating them back to their role in the ecosystem. After isolating mucin degraders and butyrate producers from the intestinal tract, characterizing their niche in the intestinal tract will help us understand the role of these functions in the gut. Designing qPCR or FISH probes specific to these bacteria (e.g. using their 16S rRNA gene and specific functional genes), will allow us to evaluate the role of these functions on niche specificity within the gut.

Bacterial communities within the pig intestine are both complex and dynamic, and by understanding how they adapt to niches or respond to perturbations, we hope to predict how bacterial communities behave in relation to host health and performance. Predicting microbial outcomes will inform potential mitigation strategies, thus leading to the ultimate objective of identifying viable alternatives to in-feed antibiotics.

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APPENDIX A. SUPPORTING INFORMATION FOR CHAPTER 2: IN-FEED ANTIBIOTIC EFFECTS ON THE SWINE INTESTINAL MICROBIOME

DNA Extractions.

Feces were processed as follows for phylotype and metagenomic analysis. Ten grams of fresh feces per sample were collected and blended in 300 ml sterile PBS. After suspension, the feces were centrifuged at 250 × g for 5 min to remove the large particles (such as insoluble food) from the sample. The supernatant was retained and centrifuged at 10,000 × g for 30 min at 4 °C to pellet the bacterial cells. The supernatant was poured off and the pellet was washed by suspending it in PBS and spinning it again at 10,000 × g for 30 min at 4 °C. Two grams of the washed pellet were used for DNA extractions using the Power Max Soil DNA Isolation Kit following the manufacturer's protocol (MO BIO Laboratories). DNA samples were quantified on a Nanodrop ND-1000 UV-Vis spectrophotometer (Nanodrop Technologies). DNA integrity was determined by gel electrophoresis. Extracted DNA was stored at −20 °C.

16S rRNA Gene Amplification.

Amplification of the V1-V3 region of bacterial 16S rRNA genes was carried out with the conserved primers 8F (5′-AGAGTTTGATCCTGGCTCAG) (1) and 518R (5′-ATTACCGCGGCTGCTGG) (2) with attached unique eight nucleotide sequence barcodes (3). The V3 region was chosen because it was shown to be highly informative (4). PCR

reactions contained 200 μM of each deoxyribonucleotide triphosphate, 2.0 μM of each primer, 2.0 U Ampligold Taq polymerase (Applied Biosystems), 2.5 mM $MgCl^2$, 50 ng template DNA, Ampligold Taq buffer (Applied Biosystems), and water to 50 μL. PCRs were performed in a PTC-225 thermal cycler (MJ Research) with the following protocol: 3 min at 95 °C, 21 cycles of (1 min at 95 °C, 30 s at 56 °C, 45 s 72 °C), and a final elongation step for 3 min at 72 °C. PCR products were separated by gel electrophoresis and purified using MinElute kit (Qiagen).

Metagenomic Analysis.

Sequence replicate artifacts were removed using a local version of the 454 Replicate Filter (5) and specifying a sequence identity cutoff of 0.9, a length difference requirement of 0, and a check for a three-base identical sequence at the beginning of each cluster. The clustered sequences were assigned to clusters of orthologous groups (COGs) by using BLASTx to compare the nucleic acid sequences to the database of proteins that was originally used to identify COGs. The BLAST reports were parsed to extract COG information, and COG frequencies were calculated and tabulated using SAS (SAS Institute). COG frequencies were subsequently analyzed in ShotgunFunctionalizeR (6) using the testGeneFamilies.dircomp function and Poisson group statistics to perform gene-centric analysis between two groups [nonmedicated (n = 3) and medicated (n = 1) swine metagenomes]. Differences with $P < 0.05$ were significant, and the significant COGs were labeled with their respective COG category to visualize trends. Metagenomic sequences belonging to select significantly different COGs were analyzed to infer phylogeny. Phylogeny assignments were made by extracting sequences belonging to the COGs of

interest, BLASTx comparison of those sequences to the GenBank nonredundant protein database, extraction of the top-hit accession, and retrieval of the phylogeny for that accession. COG counts were also corrected for differences in the estimated average genome size of each metagenome and reanalyzed as above, invoking the eff.nseq adjustment using the testGeneFamilies2.dircomp function (7). Because different methods of average genome size calculations could affect the outcome, COG counts were also corrected with the average genome sizes that were calculated by GAAS (8). These adjustments did not dramatically affect the results, and therefore only the results of the original ShotgunFunctionalizeR calculations are reported.

Swine metagenomes were also examined for the presence of known antibiotic resistance genes. MG-RAST (9) was used to bin sequences by subsystems. In addition, sequences were locally analyzed by BLASTx comparison of the sequences against the Antibiotic Resistance Gene Database (ARDB) (10), which was kindly provided by the ARDB authors. The BLASTx parameters were optimized for short reads and diversity by using a bitscore cutoff of ≥60 and an identity cutoff of 35%. Antibiotic resistance gene-centric analysis was carried out in R using the testGene-Families function as described above. Differences with $P < 0.05$ were significant. For ecological analyses, the number of hits was normalized to 100,000 submitted reads and analyzed using multidimensional scaling (MDS) and cluster analyses with the Bray-Curtis similarity measurement in PAST (11).

Design of Primers for Quantitative PCR Targeting Antibiotic Resistance Genes in Biotrove Array

Antibiotic resistance-gene reference sequences were collected using: (i) the Antibiotic Resistance Genes Online database, which contained 555 β-lactamase and 115 vancomycin

resistance-gene sequences at the time of collection (12); (ii) a National Center for Biotechnology Information (NCBI) search for resistance-gene sequences; and (iii) literature search. Reference sequence protein IDs were used as seeds to harvest all closely related alleles from GenBank using the Fun-Gene pipeline and repository (FGPR) (http://fungene.cme.msu.edu/index.spr). Aligned sequences from the FGPR were used to create consensus sequences using BioEdit (13). Primer sets were designed from consensus sequences and then selected or rejected following criteria previously described (14). Overall, 174 antibiotic resistance genes were targeted with 272 primer sets designed from 5,241 sequences. Primer sets were grouped into 18 resistance types by subjecting all primer sets to the ARDB (10) BLAST tool (Table A4), or by the BLAST tool in the NCBI when no results were obtained by the ARDB BLAST (Table A5). Abundance of the resistance type was the sum of individual genes within the resistance type. Antibiotic resistance gene categories used to group the results include (family: type): beta-lactamase: (i) class A, (ii) class B, and (iii) class C; tetracycline resistance: (iv) ribosome protection protein and (v) tetracycline efflux; (vi) sulfonamide resistance; macrolide-lincosamide-streptogramin B resistance: (vii) erm rRNA methylases, (viii) ATP-binding transporters, (ix) major facilitator family transporters, (x) hydrolases, and (xi) transferases; aminoglycoside resistance: (xii) acetylation, (xiii) adenylylation, and (xiv) phosphorylation; multidrug transporters: (xv) multidrug and toxic compound extrusion family, (xvi) major facilitator superfamily transporter, (xvii) resistance-nodulation-cell division transporter, and (xviii) small multidrug resistance transporter.

Validation of the BioTrove System for Quantitative PCR of Antibiotic Resistance Genes.

Randomly-selected genes were tested in parallel with published PCR primers that

target the same gene (Table A6). If no amplification curve was observed using the previously published primer set or if the threshold cycle was high (greater than 35), the results were confirmed further by running the quantitative PCR (qPCR) product on a 1% agarose gel and confirming presence or absence of the gene by visualization of a band of the correct length.

Validation of the BioTrove Antibiotic Resistance Genes Primer Set.

Results obtained using the BioTrove platform were validated by probing samples in parallel with primers that were previously published. Antibiotic resistance genes were randomly selected for this validation insofar as a published qPCR primer set using SYBR as the dye could be obtained. Samples were probed by qPCR and in some cases by traditional PCR and gel imaging. The result obtained using the BioTrove platform was considered "true" if the previously published primer set confirmed the result. In validation of the results, in total, there were 29 instances of true positives, 46 instances of true negatives, 2 instances of false-positives, and 7 instances of false-negatives; these results translate to an 89% success rate, which we consider satisfactory. We used strict interpretation of the PCR to determine if the BioTrove platform result was accurate or not. For example, three of the false-negative instances resulted in a very faint band on a gel, or a high threshold cycle (14). It is possible that the BioTrove primers did not fail in these instances but were simply less sensitive than other PCR reactions because of the small reaction volume. We also observed that some of the BioTrove individual primer sets may be much more broad than previously published primer sets.

Culturing Escherichia coli.

The antibiotic feed trial was repeated with an independent set of pigs. Twelve pigs (offspring from three sows) were housed and maintained as described above. Six pigs received antibiotics (ASP250) and six receive no antibiotics continuously for 21 d before being sampled. *E. coli* was cultured from fresh pig intestinal contents at necropsy, from both medicated and nonmedicated animals, after 21 d of feed. Serial dilutions were plated on MacConkey plates with lactose and incubated overnight at 39 °C. Colony forming units were enumerated for each animal (Figure A2).

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Figure A1. *E. coli* enumerations from swine gut contents in a repeated ASP250 study. *E. coli* was cultured on MacConkey's agar from fresh gut contents, from both medicated and control animals after 21 days of feed. Wide black horizontal bars show the average cfu per treatment group, which are significantly different (p=0.04).

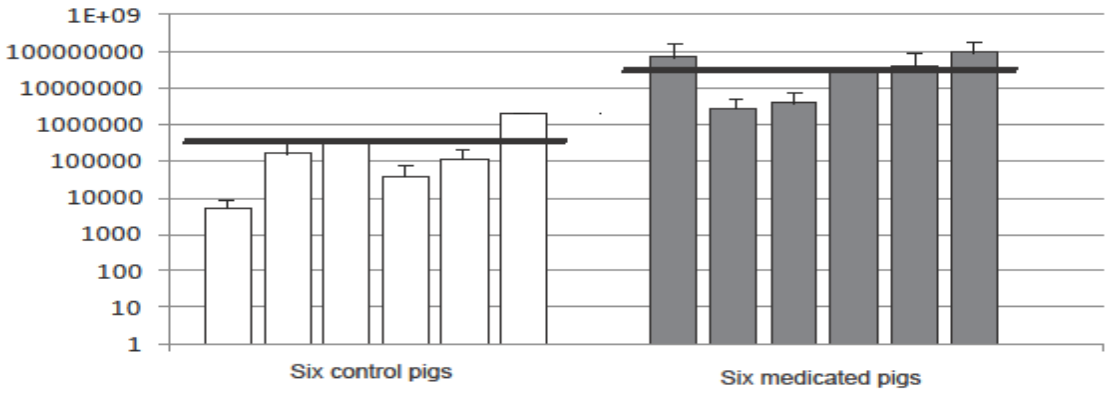

Figure A2. Microbial functions encoded by the swine metagenomes. (A) COGs in the metagenomes. The following COGs were less than 0.02% of the total number of COGs per metagenome and therefore cannot be visualized on the graph: A, RNA processing and modification; B, chromatin structure and dynamics; W, extracellular structures; Y, nuclear structure; Z, cytoskeleton. (B) SEED subsystems in the metagenomes.

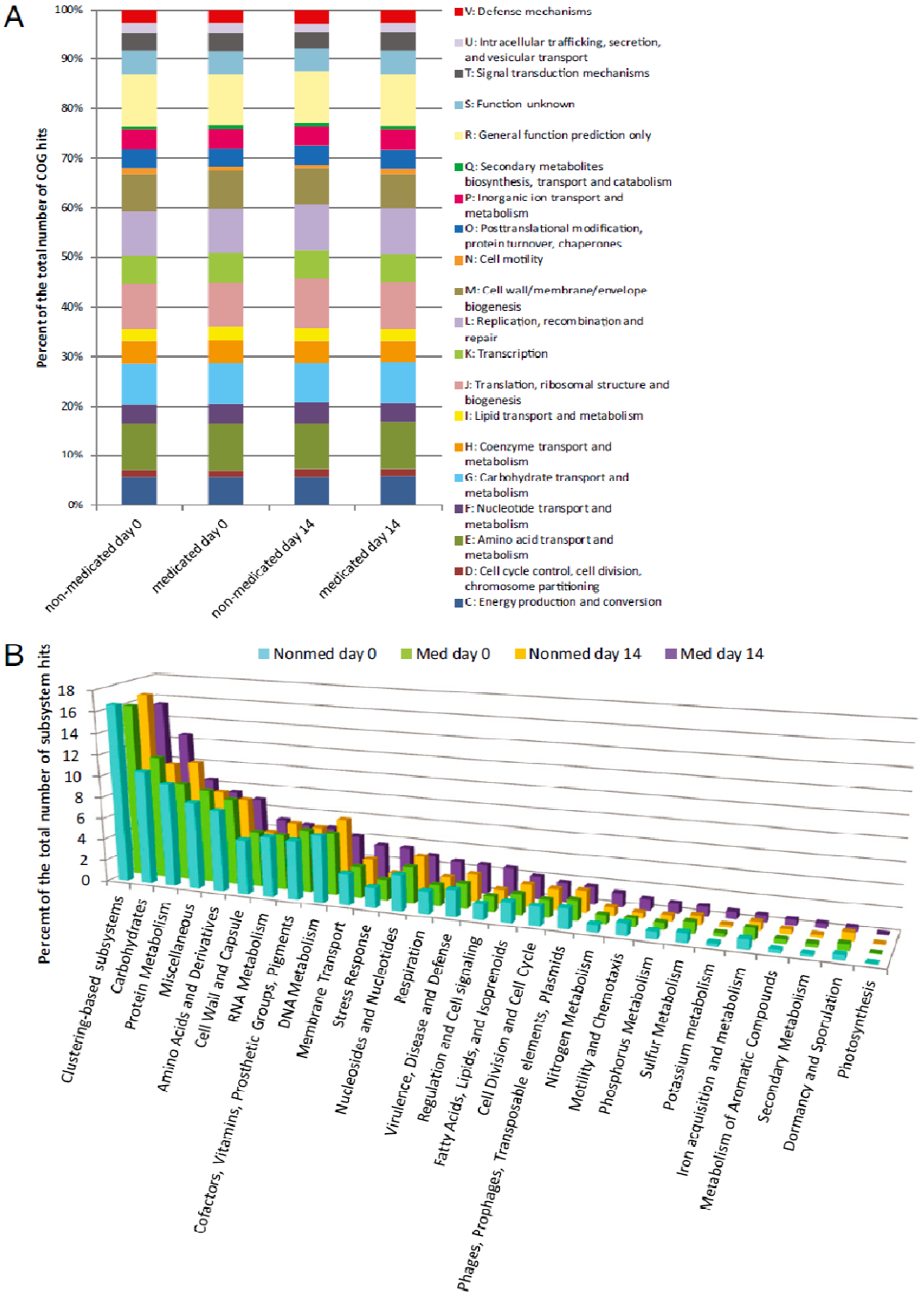

Figure A3. Tetracycline efflux abundance trends for each treatment animal. This is a representative figure; similar trends were observed for each of the six treatment-enriched gene types. Black lines are medicated animals and gray dashed lines are nonmedicated animals.

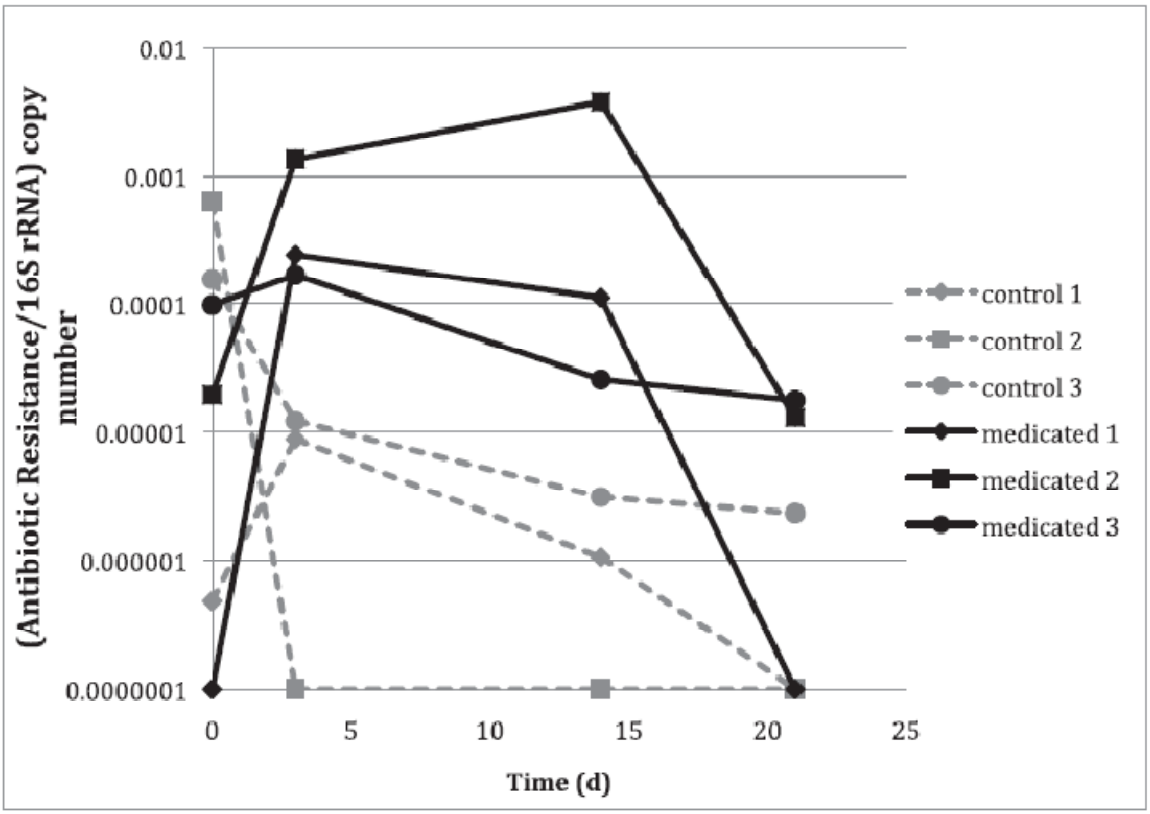

Table A1. Phylotypes based on 16S analysis, by treatment. Genus assignments were made using RDP's sequence classifier.

	medicated_0		medicated_14		control_0		control_14	
Genera	**No. of hits**	**(%)**	**No. of hits**	**(%)**	**No. of hits**	**(%)**	**No. of hits**	**(%)**
Prevotella	7479	**21.7**	8724	**29.6**	10490	**26.8**	7659	**21.4**
Succinivibrio	2901	**8.4**	1789	**6.1**	4211	**10.7**	772	**2.2**
Escherichia	256	**0.7**	3253	**11.0**	186	**0.5**	18	**0.1**
Anaerovibrio	3071	**8.9**	256	**0.9**	334	**0.9**	470	**1.3**
Selenomonas	17	**0.0**	17	**0.1**	4273	**10.9**	8	**0.0**
Coprococcus	450	**1.3**	929	**3.1**	681	**1.7**	713	**2.0**
Oscillibacter	824	**2.4**	610	**2.1**	634	**1.6**	752	**2.1**
Parabacteroides	802	**2.3**	442	**1.5**	523	**1.3**	322	**0.9**
Anaerobacter	374	**1.1**	127	**0.4**	380	**1.0**	728	**2.0**
Hallella	625	**1.8**	127	**0.4**	446	**1.1**	281	**0.8**
Roseburia	157	**0.5**	365	**1.2**	255	**0.7**	271	**0.8**
Barnesiella	136	**0.4**	135	**0.5**	316	**0.8**	473	**1.3**
Papillibacter	52	**0.2**	75	**0.3**	344	**0.9**	444	**1.2**
Treponema	203	**0.6**	137	**0.5**	165	**0.4**	286	**0.8**
Ruminococcus	91	**0.3**	204	**0.7**	83	**0.2**	64	**0.2**
Butyricicoccus	43	**0.1**	239	**0.8**	47	**0.1**	66	**0.2**
Tannerella	74	**0.2**	40	**0.1**	181	**0.5**	99	**0.3**
Paraprevotella	124	**0.4**	98	**0.3**	66	**0.2**	50	**0.1**
Subdoligranulum	59	**0.2**	131	**0.4**	135	**0.3**	13	**0.0**
Streptococcus	64	**0.2**	29	**0.1**	90	**0.2**	172	**0.5**
Phascolarctobacterium	92	**0.3**	45	**0.2**	132	**0.3**	69	**0.2**
Lactobacillus	1	**0.0**	253	**0.9**	10	**0.0**	19	**0.1**
Faecalibacterium	15	**0.0**	20	**0.1**	298	**0.8**	4	**0.0**
Bacteroides	42	**0.1**	90	**0.3**	62	**0.2**	42	**0.1**
Blautia	21	**0.1**	115	**0.4**	63	**0.2**	18	**0.1**
Butyricimonas	22	**0.1**	31	**0.1**	79	**0.2**	89	**0.2**
Akkermansia	158	**0.5**	0	**0.0**	60	**0.2**	1	**0.0**

Table A1 continued.

Dorea	17	**0.0**	88	**0.3**	49	**0.1**	46	**0.1**
Acetanaerobacterium	11	**0.0**	104	**0.4**	43	**0.1**	24	**0.1**
Sporacetigenium	67	**0.2**	6	**0.0**	56	**0.1**	54	**0.2**
Turicibacter	41	**0.1**	8	**0.0**	40	**0.1**	75	**0.2**
Clostridium	14	**0.0**	24	**0.1**	40	**0.1**	77	**0.2**
Anaerotruncus	12	**0.0**	50	**0.2**	16	**0.0**	69	**0.2**
Xylanibacter	35	**0.1**	26	**0.1**	72	**0.2**	9	**0.0**
Schwartzia	23	**0.1**	23	**0.1**	85	**0.2**	12	**0.0**
TM7_genera_incertae_sedis	2	**0.0**	61	**0.2**	3	**0.0**	32	**0.1**
Anaerostipes	12	**0.0**	61	**0.2**	16	**0.0**	8	**0.0**
Lachnobacterium	46	**0.1**	29	**0.1**	21	**0.1**	2	**0.0**
Parasporobacterium	12	**0.0**	25	**0.1**	1	**0.0**	54	**0.2**
Catenibacterium	7	**0.0**	50	**0.2**	22	**0.1**	3	**0.0**
Acetivibrio	11	**0.0**	16	**0.1**	44	**0.1**	14	**0.0**
Eubacterium	8	**0.0**	22	**0.1**	20	**0.1**	22	**0.1**
Anaerofilum	4	**0.0**	18	**0.1**	27	**0.1**	16	**0.0**
Peptostreptococcus	11	**0.0**	16	**0.1**	7	**0.0**	29	**0.1**
Campylobacter	1	**0.0**	9	**0.0**	2	**0.0**	48	**0.1**
Fibrobacter	35	**0.1**	3	**0.0**	3	**0.0**	18	**0.1**
Sarcina	4	**0.0**	0	**0.0**	20	**0.1**	34	**0.1**
Desulfovibrio	7	**0.0**	31	**0.1**	1	**0.0**	7	**0.0**
Pyramidobacter	16	**0.0**	5	**0.0**	25	**0.1**	5	**0.0**
Mitsuokella	0	**0.0**	30	**0.1**	12	**0.0**	1	**0.0**
Pseudobutyrivibrio	4	**0.0**	19	**0.1**	9	**0.0**	7	**0.0**
Spirochaeta	2	**0.0**	16	**0.1**	10	**0.0**	11	**0.0**
Rhodopirellula	2	**0.0**	9	**0.0**	0	**0.0**	25	**0.1**
Oribacterium	7	**0.0**	3	**0.0**	17	**0.0**	11	**0.0**
Anaeroglobus	0	**0.0**	0	**0.0**	1	**0.0**	35	**0.1**

Table A2. Clusters of orthologous groups (COGs) that are differentially represented in the ASP250 (n=1) vs. unmedicated (n=3) swine fecal metagenomes.

Gene Family	Adjusted P-value[a]	Annotation
More prevalent in medicated metagenome		
COG3188	0.000	P pilus assembly protein, porin PapC
COG3539	0.000	P pilus assembly protein, pilin FimA
COG0243	0.000	Anaerobic dehydrogenases, typically selenocysteine-containing
COG0814	0.000	Amino acid permeases
COG1289[b]	0.000	Predicted membrane protein
COG0675	0.000	Transposase and inactivated derivatives
COG2909	0.000	ATP-dependent transcriptional regulator
COG1662	0.000	Transposase and inactivated derivatives, IS1 family
COG4644	0.000	Transposase and inactivated derivatives, TnpA family
COG3121	0.000	P pilus assembly protein, chaperone PapD
COG3468	0.000	Type V secretory pathway, adhesin AidA
COG3570[b]	0.000	Streptomycin 6-kinase
COG3164	0.000	Predicted membrane protein
COG0430	0.000	RNA 3'-terminal phosphate cyclase
COG5013	0.000	Nitrate reductase alpha subunit
COG3596	0.000	Predicted GTPase
COG3765	0.000	Chain length determinant protein
COG0786	0.000	Na+/glutamate symporter
COG3203	0.000	Outer membrane protein (porin)
COG0583	0.000	Transcriptional regulator
COG1735	0.000	Predicted metal-dependent hydrolase with the TIM-barrel fold
COG1012	0.000	NAD-dependent aldehyde dehydrogenases
COG3623	0.000	Putative L-xylulose-5-phosphate 3-epimerase
COG2225	0.000	Malate synthase
COG4533	0.000	ABC-type uncharacterized transport system, periplasmic component
COG3019	0.000	Predicted metal-binding protein
COG3272	0.000	Uncharacterized conserved protein
COG1113	0.000	Gamma-aminobutyrate permease and related permeases
COG4943	0.000	Predicted signal transduction protein containing sensor and EAL domains
COG3131	0.000	Periplasmic glucans biosynthesis protein
COG5518	0.000	Bacteriophage capsid portal protein
COG1961	0.000	Site-specific recombinases, DNA invertase Pin homolog

Table A2 continued.

COG3850	0.000	Signal transduction histidine kinase, nitrate/nitrite-specific
COG1494	0.000	Fructose-1,6-bisphosphatase/sedoheptulose 1,7-bisphosphatase and related proteins
COG1659	0.000	Uncharacterized protein, linocin/CFP29 homolog
COG3228	0.000	Uncharacterized protein conserved in bacteria
COG1475	0.001	Predicted transcriptional regulators
COG1018	0.001	Flavodoxin reductases (ferredoxin-NADPH reductases) family 1
COG1288	0.001	Predicted membrane protein
COG2959	0.001	Uncharacterized enzyme of heme biosynthesis
COG3302	0.001	DMSO reductase anchor subunit
COG3634	0.001	Alkyl hydroperoxide reductase, large subunit
COG0654	0.001	2-polyprenyl-6-methoxyphenol hydroxylase and related FAD-dependent oxidoreductases
COG1986	0.002	Uncharacterized conserved protein
COG2352	0.002	Phosphoenolpyruvate carboxylase
COG1140	0.002	Nitrate reductase beta subunit
COG2116	0.002	Formate/nitrite family of transporters
COG1298	0.003	Flagellar biosynthesis pathway, component FlhA
COG1391	0.003	Glutamine synthetase adenylyltransferase
COG0064	0.004	Asp-tRNAAsn/Glu-tRNAGln amidotransferase B subunit (PET112 homolog)
COG0754	0.004	Glutathionylspermidine synthase
COG5339	0.004	Uncharacterized protein conserved in bacteria
COG2837	0.004	Predicted iron-dependent peroxidase
COG3477	0.005	Predicted periplasmic/secreted protein
COG5404	0.005	SOS-response cell division inhibitor, blocks FtsZ ring formation
COG5463	0.005	Predicted integral membrane protein
COG5562	0.005	Phage envelope protein
COG1172	0.005	Ribose/xylose/arabinose/galactoside ABC-type transport systems, permease components
COG2186	0.005	Transcriptional regulators
COG2844	0.005	UTP:GlnB (protein PII) uridylyltransferase
COG4509	0.005	Uncharacterized protein conserved in bacteria
COG1887	0.005	Putative glycosyl/glycerophosphate transferases involved in teichoic acid biosynthesis TagF/TagB/EpsJ/RodC
COG1276	0.005	Putative copper export protein
COG3626	0.005	Uncharacterized enzyme of phosphonate metabolism
OG2375	0.005	Siderophore-interacting protein

Table A2 continued

COG3414	0.005	Phosphotransferase system, galactitol-specific IIB component
COG5532	0.005	Uncharacterized conserved protein
COG1199	0.005	Rad3-related DNA helicases
COG2937	0.006	Glycerol-3-phosphate O-acyltransferase
COG0531	0.006	Amino acid transporters
COG3264	0.007	Small-conductance mechanosensitive channel
COG1454	0.007	Alcohol dehydrogenase, class IV
COG2515	0.007	1-aminocyclopropane-1-carboxylate deaminase
COG3768	0.007	Predicted membrane protein
COG2513	0.008	PEP phosphonomutase and related enzymes
COG0709	0.008	Selenophosphate synthase
COG0477[b]	0.008	Permeases of the major facilitator superfamily
COG1988	0.008	Predicted membrane-bound metal-dependent hydrolases
COG3449	0.008	DNA gyrase inhibitor
COG2224	0.008	Isocitrate lyase
COG2943	0.008	Membrane glycosyltransferase
COG0321	0.008	Lipoate-protein ligase B
COG3209	0.009	Rhs family protein
COG2336	0.009	Growth regulator
COG4573	0.009	Predicted tagatose 6-phosphate kinase
COG2813	0.009	16S RNA G1207 methylase RsmC
COG3316	0.010	Transposase and inactivated derivatives
COG1131	0.011	ABC-type multidrug transport system, ATPase component
COG4172	0.011	ABC-type uncharacterized transport system, duplicated ATPase component
COG1982	0.011	Arginine/lysine/ornithine decarboxylases
COG1832	0.011	Predicted CoA-binding protein
COG3078	0.011	Uncharacterized protein conserved in bacteria
COG3685	0.011	Uncharacterized protein conserved in bacteria
COG3700	0.011	Acid phosphatase (class B)
COG4700	0.011	Uncharacterized protein conserved in bacteria containing a divergent form of TPR repeats
COG0666	0.012	FOG: Ankyrin repeat
COG3691	0.012	Uncharacterized protein conserved in bacteria
COG3971	0.012	2-keto-4-pentenoate hydratase
COG2963	0.012	Transposase and inactivated derivatives
COG3293	0.012	Transposase and inactivated derivatives
COG0031	0.013	Cysteine synthase
OG3605	0.013	Signal transduction protein containing GAF and PtsI domains
COG2182	0.016	Maltose-binding periplasmic proteins/domains

Table A2 continued

COG4580	0.016	Maltoporin (phage lambda and maltose receptor)
COG3954	0.017	Phosphoribulokinase
COG3054	0.017	Predicted transcriptional regulator
COG1686	0.017	D-alanyl-D-alanine carboxypeptidase
COG4579	0.018	Isocitrate dehydrogenase kinase/phosphatase
COG4134	0.018	ABC-type uncharacterized transport system, periplasmic component
COG0745	0.018	Response regulators consisting of a CheY-like receiver domain and a winged-helix DNA-binding domain
COG2933	0.018	Predicted SAM-dependent methyltransferase
COG3025	0.018	Uncharacterized conserved protein
COG5410	0.018	Uncharacterized protein conserved in bacteria
COG2205	0.019	Osmosensitive K+ channel histidine kinase
COG0449	0.021	Glucosamine 6-phosphate synthetase, contains amidotransferase and phosphosugar isomerase domains
COG2080	0.021	Aerobic-type carbon monoxide dehydrogenase, small subunit CoxS/CutS homologs
COG1201	0.023	Lhr-like helicases
COG2961	0.023	Protein involved in catabolism of external DNA
COG4565	0.023	Response regulator of citrate/malate metabolism
COG1263	0.023	Phosphotransferase system IIC components, glucose/maltose/N-acetylglucosamine-specific
COG3096	0.023	Uncharacterized protein involved in chromosome partitioning
COG2739	0.023	Uncharacterized protein conserved in bacteria
COG1882	0.025	Pyruvate-formate lyase
COG2088	0.025	Uncharacterized protein, involved in the regulation of septum location
COG1357	0.026	Uncharacterized low-complexity proteins
COG1250	0.026	3-hydroxyacyl-CoA dehydrogenase
COG0825	0.028	Acetyl-CoA carboxylase alpha subunit
COG1972	0.028	Nucleoside permease
COG0818	0.029	Diacylglycerol kinase
COG1732	0.029	Periplasmic glycine betaine/choline-binding (lipo)protein of an ABC-type transport system (osmoprotectant binding protein)
COG2918	0.029	Gamma-glutamylcysteine synthetase
COG3261	0.030	Ni,Fe-hydrogenase III large subunit
OG2223	0.030	Nitrate/nitrite transporter
COG4776	0.030	Exoribonuclease II
COG3061	0.030	Cell envelope opacity-associated protein A
COG3840	0.030	ABC-type thiamine transport system, ATPase component

Table A2 continued

COG1780	0.030	Protein involved in ribonucleotide reduction
COG2862	0.030	Predicted membrane protein
COG2924	0.030	Fe-S cluster protector protein
COG3076	0.030	Uncharacterized protein conserved in bacteria
COG3097	0.030	Uncharacterized protein conserved in bacteria
COG3257	0.030	Uncharacterized protein, possibly involved in glyoxylate utilization
COG3592	0.030	Uncharacterized conserved protein
COG3783	0.030	Soluble cytochrome b562
COG4135	0.030	ABC-type uncharacterized transport system, permease component
COG0702	0.030	Predicted nucleoside-diphosphate-sugar epimerases
COG2995	0.031	Uncharacterized paraquat-inducible protein A
COG0148	0.031	Enolase
COG0641	0.036	Arylsulfatase regulator (Fe-S oxidoreductase)
COG2180	0.036	Nitrate reductase delta subunit
COG2717	0.036	Predicted membrane protein
COG4685	0.036	Uncharacterized protein conserved in bacteria
COG0364	0.037	Glucose-6-phosphate 1-dehydrogenase
COG1076	0.037	DnaJ-domain-containing proteins 1
COG2915	0.037	Uncharacterized protein involved in purine metabolism
COG3775	0.037	Phosphotransferase system, galactitol-specific IIC component
COG2704	0.038	Anaerobic C4-dicarboxylate transporter
COG2864	0.038	Cytochrome b subunit of formate dehydrogenase
COG0121	0.038	Predicted glutamine amidotransferase
COG2269	0.038	Truncated, possibly inactive, lysyl-tRNA synthetase (class II)
COG2161	0.038	Antitoxin of toxin-antitoxin stability system
COG2895	0.039	GTPases - Sulfate adenylate transferase subunit 1
COG1064	0.040	Zn-dependent alcohol dehydrogenases
COG0433	0.042	Predicted ATPase
COG0815	0.042	Apolipoprotein N-acyltransferase
COG4932	0.042	Predicted outer membrane protein
COG1593	0.045	TRAP-type C4-dicarboxylate transport system, large permease component
OG0695	0.045	Glutaredoxin and related proteins
COG1636	0.045	Uncharacterized protein conserved in bacteria
More prevalent in control metagenomes		
COG1629	0.000	Outer membrane receptor proteins, mostly Fe transport

Table A2 continued

COG1672	0.000	Predicted ATPase (AAA+ superfamily)
COG1506	0.000	Dipeptidyl aminopeptidases/acylaminoacyl-peptidases
COG0457	0.000	FOG: TPR repeat
COG1373	0.000	Predicted ATPase (AAA+ superfamily)
COG3385	0.000	FOG: Transposase and inactivated derivatives
COG0438	0.000	Glycosyltransferase
COG3292	0.000	Predicted periplasmic ligand-binding sensor domain
COG0463	0.000	Glycosyltransferases involved in cell wall biogenesis
COG1216	0.000	Predicted glycosyltransferases
COG3210	0.000	Large exoproteins involved in heme utilization or adhesion
COG3344	0.000	Retron-type reverse transcriptase
COG0514	0.000	Superfamily II DNA helicase
COG0614	0.001	ABC-type Fe3+-hydroxamate transport system, periplasmic component
COG1260	0.001	Myo-inositol-1-phosphate synthase
COG1205	0.001	Distinct helicase family with a unique C-terminal domain including a metal-binding cysteine cluster
COG1649	0.001	Uncharacterized protein conserved in bacteria
COG3666	0.002	Transposase and inactivated derivatives
COG3876	0.002	Uncharacterized protein conserved in bacteria
COG2148	0.003	Sugar transferases involved in lipopolysaccharide synthesis
COG1086	0.003	Predicted nucleoside-diphosphate sugar epimerases
COG3039	0.004	Transposase and inactivated derivatives, IS5 family
COG2875	0.004	Precorrin-4 methylase
COG0732	0.005	Restriction endonuclease S subunits
COG3589	0.006	Uncharacterized conserved protein
COG1522	0.007	Transcriptional regulators
COG1595	0.007	DNA-directed RNA polymerase specialized sigma subunit, sigma24 homolog
COG1453	0.008	Predicted oxidoreductases of the aldo/keto reductase family
COG1479	0.008	Uncharacterized conserved protein
COG4412	0.010	Uncharacterized protein conserved in bacteria
COG0153	0.011	Galactokinase
COG1472	0.011	Beta-glucosidase-related glycosidases
COG0526	0.011	Thiol-disulfide isomerase and thioredoxins
COG3177	0.013	Uncharacterized conserved protein
COG4823	0.013	Abortive infection bacteriophage resistance protein
COG1055	0.013	Na+/H+ antiporter NhaD and related arsenite permeases
COG1404	0.014	Subtilisin-like serine proteases
COG2319	0.015	FOG: WD40 repeat

Table A2 continued

COG3914	0.017	Predicted O-linked N-acetylglucosamine transferase, SPINDLY family
COG4463	0.019	Transcriptional repressor of class III stress genes
COG0615	0.019	Cytidylyltransferase
COG3882	0.023	Predicted enzyme involved in methoxymalonyl-ACP biosynthesis
COG2865	0.024	Predicted transcriptional regulator containing an HTH domain and an uncharacterized domain shared with the mammalian protein Schlafen
COG4898	0.025	Uncharacterized protein conserved in bacteria
COG4372	0.027	Uncharacterized protein conserved in bacteria with the myosin-like domain
COG0840	0.028	Methyl-accepting chemotaxis protein
COG3590	0.028	Predicted metalloendopeptidase
COG3600	0.028	Uncharacterized phage-associated protein
COG5368	0.029	Uncharacterized protein conserved in bacteria
COG4690	0.030	Dipeptidase
COG1508	0.032	DNA-directed RNA polymerase specialized sigma subunit, sigma54 homolog
COG0302	0.032	GTP cyclohydrolase I
COG2189	0.032	Adenine specific DNA methylase Mod
COG1188	0.036	Ribosome-associated heat shock protein implicated in the recycling of the 50S subunit (S4 paralog)
COG3176	0.038	Putative hemolysin
COG0535	0.038	Predicted Fe-S oxidoreductases
COG4206	0.038	Outer membrane cobalamin receptor protein
COG3878	0.038	Uncharacterized protein conserved in bacteria
COG3527	0.038	Alpha-acetolactate decarboxylase
COG3560	0.038	Predicted oxidoreductase related to nitroreductase
COG1088	0.041	dTDP-D-glucose 4,6-dehydratase
COG3291	0.042	FOG: PKD repeat
COG1022	0.044	Long-chain acyl-CoA synthetases (AMP-forming)

a. The *P*-value cut-off was <0.05. *P*-values are adjusted for multiple testing. See Kristiansson et al. 2010 for more details.

b. These COGs contain genes that were also identified in the antibiotic resistance gene database (Liu and Pop 2009) (Table 2.2).

Table A3. Individual COGs of the energy production and conversion COG category that were significantly more prevalent in the medicated metagenome (n = 1) than the nonmedicated metagenomes (n = 3)

Gene family	Description
COG0243	Anaerobic dehydrogenases, typically selenocysteine-containing
COG1012	NAD-dependent aldehyde dehydrogenases
COG1018	Flavodoxin reductases (ferredoxin-NADPH reductases) family 1
COG1140	Nitrate reductase beta subunit
COG1454	Alcohol dehydrogenase, class IV
COG1882	Pyruvate-formate lyase
COG2080	Aerobic-type carbon monoxide dehydrogenase, small subunit CoxS/CutS homologs
COG2180	Nitrate reductase delta subunit
COG2224	Isocitrate lyase
COG2225	Malate synthase
COG2352	Phosphoenolpyruvate carboxylase
COG2864	Cytochrome b subunit of formate dehydrogenase
COG2924	Fe-S cluster protector protein
COG3261	Ni,Fe-hydrogenase III large subunit
COG3783	Soluble cytochrome b562
COG3954	Phosphoribulokinase
COG5013	Nitrate reductase alpha subunit

Table A4. Primer sets targeting antibiotic resistance genes (specificity classified by ARDB)

Forward Primer	Reverse Primer	ARG name
CGTCACTTATTCGATGCCCTTAC	GTCGGGCGCGGCATA	aac3vi
GTTTGAGAGGCAAGGTACCGTAA	GAATGCCTGGCGTGTTTGA	aac6ib
ACACTGCGCCTCATGACTGA	GTCGGGCGTGCTTCTTCTC	aac6ib
AGAAGCACGCCCGACACTT	GCTCTCCATTCAGCATTGCA	aac6ib
CCGACAACATTTCTACCATCCTT	ACCGAAGCGCTCGTCGTATA	aadd
GGTCTATCACCCTACGCGCTATC	GCGCGCACGAACATACC	acra
CAACGATCGGACGGGTTTC	TGGCGATGCCACCGTACT	acra
TACTTTGCGCGCCATCTTC	CGTGCGCGAACGAACAT	acra
ACGGCTCCGCAGTGGAT	GGCCACAGTAACCAACAAATCA	ant2ia
CTTGTCGTGCATGACGACATC	TCGAAGATACCCGCAAGAATG	ant2ia
AGCTAAGCGCGAACTGCAAT	TGGCTCGAAGATACCTGCAA	ant3ia
GTTGTGCACGACGACATCATT	GGCTCGAAGATACCTGCAAGAA	ant3ia
CAATGACATTCTTGCGGGTATC	GACCTACCAAGGCAACGCTATG	ant3ia
CGAGATTCTCCGCGCTGTA	GCTGCCATTCTCCAAATTGC	ant3ia
TTGTGCACGACGACATCATTC	TGGCTCGAAGATACCTGCAA	ant3ia
TGAACAAGTCTGGAAAGAAATGCA	CCTATTAATTTCCCCTCGTCAAAAA	aph3ia
CGGAATTGAAAAAACTGATCGAA	ATACCGGCTGTCCGTCATTT	aph3iiia
GCTCGGTCGTGAGAACAATCT	CAATTTCGGTCGCCTGGTAGT	aph6id
TTTTGGGTGTGGTCGAAGGA	GGCCGTATCGCCTTCAAAC	baca
TTCCACGACACGATTAAGTCATTG	CGGCTCTTTCGGCTTCAG	baca
CCGGATGAGGTCACGGATAC	ATGCTGGCGTTAGCGTAAAGA	bl1_ampc
CCGCTCAAGCTGGACCATAC	CCATATCCTGCACGTTGGTTT	bl1_ampc
CTGTTCGAGCTGGGTTCTATAAGTAAA	CAGTATCTGGTCACCGGATCGT	bl1_ampc
AAAGCCTCATGGGTGCATAAA	ATAGCTTTTGTTTGCCAGCATCA	bl1_cmy2
CAGCCGCTGATGAAAAAATATG	CAGCGAGCCCACTTCGA	bl1_sm
GCAATGTGCTCAACGTTCAAG	GTGCCTGAGTCAATTCTTTCAAAG	bl2_ges
GGAGATAAAGTAACAAATCCAGTTAGATATGA	TGCTTAATTTTCCATTTGCGATAAG	bl2a_pc
TTGACGCCGGGCAAGA	TGCTTTTCTGTGACTGGTGAGTACT	bl2b_tem1
AGCATCTTACGGATGGCATGA	TCCTCCGATCGTTGTCAGAAGT	bl2b_tem1
CTGATTGACACCGCCGATAAC	GGTGCTGCACATGGCAAAG	bl2be_ctxm
CCGCCATGAACAAATTGATTG	TTCAGTGCGATCCAGACGAA	bl2be_ctxm

Table A4 continued

GCGATAACGTGGCGATGAAT	GTCGAGACGGAACGTTTCGT	bl2be_ctxm
CACAGTTGGTGACGTGGCTTAA	CTCCGCTGCCGGTTTTATC	bl2be_ctxm
TGCTGGTTGCTGTTTTTGTGA	CCTGCGCAATGATAGCTTCAT	bl2be_per
GGCGCGCCGATGAAC	TCCTGCTGGCGATAGTGGAT	bl2be_shv2
CTTTCCCATGATGAGCACCTTT	TCCTGCTGGCGATAGTGGAT	bl2be_shv2
CGGATGGTTTGAAGGGTTTATTAT	TCTTGGCTTTTATGCTTGATGTTAA	bl2d_oxa1
AGTTGCGCAGAACAGTCCTCTT	TCGTATCTTGCCCGTCGATAAT	bl2e_cepa
TCATTCCTCGTTCAAGTTTTCAGA	TGCAGCACCAAGAGGAGATGT	bl2e_cfxa
CCGCCAAATTATTAATGTCCAAAT	CAAGAGTGATGCGTCTCCAACTT	bl3_imp
AACACGGTTTGGTGGTTCTTGTA	GCGCTCCACAAACCAATTG	bl3_imp
AAGGCAGCATTTCCTCTCATTTT	GGATAGATCGAGAATTAAGCCACTCT	bl3_imp
GCACTTCTCGCGGAGATTG	CGACGGTGATGCGTACGTT	bl3_vim
GGGTGAGTTTCACCAGTTTTGATT	CACCTTGTCGCCTTGCGTATA	cata1
TAGGAAGCATCGGAACGTTGAT	CAGACCGAGCACGACTGTTG	cml_e1
AGGAAGCATCGGAACGTTGA	ACAGACCGAGCACGACTGTTG	cml_e1
CCTGTGGTACGGAGAATTCATGT	ACCGCATTCGCTTTGCTT	erea
CGGATCAGGAAAAGGACATTTT	AGCCTCCATCAATTTCTATAGCAGTAA	erma
CATTTTACCAAGGAACTTGTGGAA	TGGCATGACATAAACCTTCATCA	erma
AAATCGGATCAGGAAAAGGACAT	CCTCCATCAATTTCTATAGCAGTAACTG	erma
TTGAGAAGGGATTTGCGAAAAG	ATATCCATCTCCACCATTAATAGTAAACC	erma
ACATTTTACCAAGGAACTTGTGGAA	GTGGCATGACATAAACCTTCATCA	erma
TGAAAGCCATGCGTCTGACA	CCCTAGTGTTCGGTGAATATCCA	ermb
ATTCACCGAACACTAGGGTTGCT	CATTCCGCTGGCAGCTTAA	ermb
TAAAGGGCATTTAACGACGAAACT	TTTATACCTCTGTTTGTTAGGGAATTGAA	ermb
GTCATCTATTCAACTTATCGTCAGAAAAAT	ACCTCTGTTTGTTAGGGAATTGAAA	ermb
CGTGGAATACGGGTTTGCTAA	TAGGATGAAAATATTCTCTTGGAACCAT	ermc
ATATCTTTGAAATCGGCTCAGGAA	ATGGTCTATTTCAATGGCAGTTACG	ermc
AATACAAAACGCTCATTGGCATT	TAGGATGAAAATATTCTCTTGGAACCATA	ermc
TTTGAAATCGGCTCAGGAAAA	ATGGTCTATTTCAATGGCAGTTACG	ermc
TTTCAAAGTGGTGTCAAATATTCCTT	GGACAATGGAACCTCCCAGAA	ermf
ATTCCTTATGGCATTACTTCCGATATT	AATGGAACCTCCCAGAAAATTTC	ermf
CAGCTTTGGTTGAACATTTACGAA	AAATTCCTAAAATCACAACCGACAA	ermf

Table A4 continued

GTGTCAAATATTCCTTATGGCATTACTT	GACAATGGAACCTCCCAGAAAAT	ermf
GTTCACTAGCACTATTTTTAATGACAGAAGT	GAAGGGTGTCTTTTTAATACAATTAACGA	ermt
GTAAAATCCCTAGAGAATACTTTCATCCA	TGAGTGATATTTTTGAAGGGTGTCTT	ermt
GCTCAGTGGTCCCCATGGT	ATCCCCCCGTCAACGTTT	ermx
CTCGGGTACCAAGGTCTGCTT	CACTAAGATCCCCCCGTCAA	ermx
TTGTCTTTGAAAGTGAAGCAACAGT	TAACGCTAGAGAACGATTTGTATTGAG	ermy
TCGACGTGACCGTAGTGAACA	CGTGACTACCCAGGTGAGTTGA	lmra
TGACGCTCAACACACTCAAAAA	TTCATGCTTAAGTTCCATACGTGAA	lnua
AGAATGAAAAAGAAGCTGAGCTTCTT	AAGGTGGCAATTACGTTTTTCAAA	lnua
TGAACATAATCCCCTCGTTTAAAGAT	TAATTGCCCTGTTTCATCGTAAATAA	lnub
AAAGGAGAAGGTGACCAATACTCTGA	GGAGCTACGTCAAACAACCAGTT	lnub
GGTTACGGACAAGGTGAAATACTGAT	TGTCTTTTAATAAGTGAGGTGCGTTAATA	meca
CCGTAGCATTGGAACAGCTTTT	AAACGGAGTATAAGAGTGCTGCAA	mefa
CGGTTACACCACTTTTAGTACCAGAAG	TGCAACTGCCGGACTAACAA	mefa
TTGCATCTATTACGGTAGCAATTGTA	GTCTCAATGCGACAATTCCTTCTT	mefa
TGCGGTTACGCCACTTTTAGTA	CAACCGCCGGACTAACAATATAG	mefa
ATGAACGGGATCGACAATCTG	GCCAGTTGCAGCTTGTTCTG	mexb
GGTCAGCACCGACAAGGTCTAC	AGCTCGACGTACTTGAGGAACAC	mexe
CCGCGAGAAGGCCAAGA	TTGAGTTCGGCGGTGATGA	mexf
CGCAGCGCTTGATCTTGTAG	TTACTGCATCCATACGCTGCTT	mphb
CGTTTGAAGTACCGAATTGGAAA	GCTGCGGGTTTGCCTGTA	mphc
CTGCTAACACAAGTACGATTCCAAAT	TCAAGTAAAGTTGTCTTACCTACACCATT	msra
AACGAAATCAAGCGCAACAA	CAACCGTGCCTTTTTCTTTTG	msra
TGGCAATAGGAGCTATGGTGTTT	AAGGTAACACTATTTTCGGTCCAAATC	qaca
AATGAGTTTTGGAGTGTCTCAACGTA	AATCAAAACCCCTATTAAAGCCAAT	str
TCATCTGCCAAACTCGTCGTTA	GTCAAAGAACGCCGCAATGT	sul2
GCTGTTTGTTCTGCCGGAAA	GGTTAAGTTCCTTGAACGCAAACT	tet30
CCATTACTTCGGACAACGGTAGA	CAATCTCTGTGAGGGCATTTAACA	tet32
CTTAGCGCAAACAGCAATCAGT	CGGTGATACAGCGCGTAAACT	tet34
AGAATACTCAGCAGAGGTCAGTTCCT	TGGTAGGTCGATAACCCGAAAAT	tet36
TGCAGGAAAGACCTCCATTACAG	CTTTGTCCACACTTCCACGTACTATG	tet36
TTAATGTGGCGGTATCTGTAGGTATT	TTGCCTGGGAAATTTAATGCTTT	tet38

Table A4 continued

CTCACCAGCCTGACCTCGAT	CACGTTGTTATAGAAGCCGCATAG	teta
AGTGCGCTTTGGATGCTGTA	AGCCCCAGTAGCTCCTGTGA	tetb
GCCCAGTGCTGTTGTTGTCAT	TGAAAGCAAACGGCCTAAATACA	tetb
TGCCGCGTTTGATTACACA	CACCAGTGATCCCGGAGATAA	tetd
TGTCATCGCGCTGGTGATT	CATCCGCTTCCGGGAGAT	tetd
TCAACCATTGCCGATTCGA	TGGCCCGGCAATCATG	tetg
CATCAGCGCCGGTCTTATG	CCCCATGTAGCCGAACCA	tetg
TTTGGGTCATCTTACCAGCATTAA	TTGCGCATTATCATCGACAGA	teth
GGGTGCCGCATTAGATTACCT	TCGTCCAATGTAGAGCATCCATA	tetj
CAGCAGTCATTGGAAAATTATCTGATTATA	CCTTGTACTAACCTACCAAAAATCAAAATA	tetk
AGCCCGATTTATTCAAGGAATTG	CAAATGCTTTCCCCCTGTTCT	tetl
ATGGTTGTAGTTGCGCGCTATAT	ATCGCTGGACCGACTCCTT	tetl
CATCATAGACACGCCAGGACATAT	CGCCATCTTTTGCAGAAATCA	tetm
TAATATTGGAGTTTTAGCTCATGTTGATG	CCTCTCTGACGTTCTAAAAGCGTATTAT	tetm
TTTTAGAACGTCAGAGAGGAATTACAAT	CGTGTCTATGATGTTCACCTTCGT	tetm
ATAAAGGCACAACAAGAACGGATT	CGGCAGCCTGAATGGTAATT	tetm
ATGTGGATACTACAACGCATGAGATT	TGCCTCCACATGATATTTTTCCT	teto
AGTTGCAGATGTGTATAGTCGTAAACTATCTATT	TGCTACAAGTACGAAAACAAAACTAGAA	tetpa
CGCCTCAGAAGTAAGTTCATACACTAAG	TCGTTCATGCGGATATTATCAGAAT	tetq
TTAAGGACAAACTTTCTGACGACATC	TGTCTCCCATTGTTCTGGTTCA	tets
GCGGGAACGACGATGTATATC	CCGCTATCTCACGACCATGAT	tetv
TCCTTCCAGTGGCACAGATGT	GCCCCATCTAAAACAGCCAAA	tetw
TTGCAGAACTAGGGAGCGTAGAT	AAAAGATGTCACTGCTGTCTGGATA	tetw
ATGAACATTCCCACCGTTATCTTT	ATATCGGCGGAGAGCTTATCC	tetw
CAACATTAACGGAAAGTTTATTGTATACCA	TTGACGCTCCAAATTCATTGTATC	tetw
AAATTTGTTACCGACACGGAAGTT	CATAGCTGAAAAAATCCAGGACAGTT	tetx
AAAAGGCTCTGAAAACGCAGTTAT	CGGCCGTTATCTTGTAAAAACAT	vana
TTGTCGGCGAAGTGGATCA	AGCCTTTTTCCGGCTCGTT	vanb
CCGGTCGAGGAACGAAATC	TCCTCCTGCAAAAAAAGATCAAC	vanb
CAGAGGAACATAATGTTTCGATAAAATCT	GCCGGATTTTGTGATTCCAA	vand
GAGGTTTCCGAGGCGACAA	CTCTCGGCGGCAGTCGTAT	vanhb
GTGGCCGATTATACCGTCATG	CGCAGGTCATTCAGGCAAT	vanhd

Table A4 continued

CCCTTACTCCCACCGAGTTTT	TTCGTCGCCCCATATCTCAT	vanra
CCACTCCGGCCTTGTCATT	GCTAACCACATTCCCCTTGTTTT	vanra
CGCGTCATGCTTTCAAAATTC	TCCGCAGAAAGCTCAATTTGTT	vansa
CGGACAAAGATACCCCCTATAAAG	AAATAGTAAATTGCTCATCTGGCACAT	vanwb
ACATTTTCATTTTGGCAGCTTGTAC	CCGCCATAAGAGCCTACAATCT	vanwg
CGCTAAATATGCCACTTGGGATA	TCAAAAGCGATTCAGCCAACT	vanxa
AGGCACAAAATCGAAGATGCTT	GGGTATGGCTCATCAATCAACTT	vanxb
TAAACCGTGTTATGGGAACGAA	GCGATAGCCGTCCCATAAGA	vanxd
TGCAATAGTAGCTGCTAATTCTGTTGTT	TGTTTTATTTCGTTAGCAGGATTTCC	vatd
GGTGCCATTATCGGAGCAAAT	TTGGATTGCCACCGACAAT	vate
GACCGTCCTACCAGGCGTAA	TTGGATTGCCACCGACAATT	vate
CGAGTATTGTGGAAAGCAGCTAGTT	CCCGTACCGTTAGAGCCGATA	vgaa
GACGGGTATTGTGGAAAGCAA	TTTCCTGTACCATTAGATCCGATAATT	vgaa
ATACGAGCTGCCTAATAAAGGATCTT	TGTGAACCACAGGGCATTATCA	vgba

Table A5. Primer sets targeting antibiotic resistance genes (specificity classified by NCBI)

Forward Primer	Reverse Primer	ARG name
CGTCGCCGAGCAACTTG	CGGTACCTTGCCTCTCAAACC	aac
CGCGGCAAGCCTATCTTG	CAAATCAGCGACCGCAGACT	aad9
GGATGCACGCTTGGATGAA	CCTCTAGCGGCCGGAGTATT	aad9
ATCACGATCTTGCGATTTTGCT	CTGCGGATGGGCCTAGAAG	aadA5
GTTCTTGCTCTTGCTCGCATT	GATGCTCGGCAGGCAAAC	aadA5
GTTCTTGCTCTTGCTCGCATT	GATGCTCGGCAGGCAAAC	aadA5
GAATGGGCAAAGCATAAAAACTTG	CCGATTTTGAAACCACAATTATGATA	aadE
CGTGCGCGAACGAACA	ACTTTGCGCGCCATCTTC	acrA
GATGATACCCCCTGCTGTGAGA	ACCAAACAAGAAGCGCAAGAA	acrA
GCGCTGGAGACACGACAAC	GCCTTGCTGCGAGAACAAA	acrA
CAGACCCGCATCGCATATT	CGACAATTTCGCGCTCATG	acrA
TTCTTGCTCTTGCTCGCATTT	GATGCTCGGCAGGCAAAC	ant4IIa
TACCTTATTGCCCTTGGAAGAGTTA	GGAACTATGTCCCTTTTAATTCTACAATCT	ant6ia
TAAGGATATACCGACAGTTTTGGAAA	TTTAATCCCTCTTCATACCAATCCATA	aph2
TGAGCAGTATCATAAGTTGAGTGAAAAG	GACAGAACAATCAATCTCTATGGAATG	aph2
CCGGTGGCATTTGAGAAAAA	GTGGCTCAACCTGCGAAAAG	aph33ib
ATACAGCAGTGGATATTGGTTTAATTGT	TGCATAAGGTGAATGTTCCATGA	araJ
CGGCTTCGTGACCTCGTT	ACAATGCGATACCAGGCAAAT	bacA
CTGCGTGTCGATGGTGTCAT	GCCGTTCCCGCCATATG	bcr
GCGGATCTCTGGTCAGCAA	TGATTGATGGTTCCCCGTACA	bexA
GGTTTGCCGCTGCAGTTC	GCGGCCAGGTGACCAA	bl1_ampc
CCGCCCAGAGCAAGGACTA	GCTCGACTTCACGCCGTAAG	bl1_ampc
GCGAGCAGCCTGAAGCA	CGGATGGGCTTGTCCTCTT	bl1_cmy2
GCAGCGAAGCGTCAGTCA	AGATCCGTGGCCGCATAA	blaBES
GGAGGCGTGACGGCTTTT	TTCAGTGCGATCCAGACGAA	blaCTX
CGATACCACCACGCCGTTA	GCATTGCCCAACGTCAGATT	blaCTX
GCCGCGGTGCTGAAGA	ATCGGATTATAGTTAACCAGGTCAGATTT	blaCTX
CTTGGCGTTGCGCTGAT	CGTTCATCGGCACGGTAGA	blaCTX
CCGCCGCCATCCAGTA	GGGCCGCCAAGATGCT	blaSFO
ACACTTTGCCATTGCTGTTTATGT	TGCAAATTTCGGCAATAATCTTT	blaTLA
CGCAACTAGCTCTGCATGACAT	CTCGCCCGGCTTGATG	carA
GGAGTGAGGCTGACCGTAGAAG	ATCGGCGAAACGCACAAA	carB

Table A5 contiued

GCAGCGTTGCTGGACACA	GTTCGGGATAAACGTGGTGACT	cfiA
GCAGCGTTGCTGGACACA	TTCGGGATAAACGTGGTGACTT	cfiA
AGGTTCGGACTCAATGCAACA	TTCAGCACATACCGCCTTGAT	cfiA
GGAAATCAACGGCATCCAGTT	CATCCATGCGCTTTTGTCTCT	cfiA
GCAAAATTCAGAGCAAGTTACGAA	AAAATGACTCCCAACCTGCTTTAT	cfr
GCAGCAAAGAAGAAGCACCAA	AGCAGGGTAAGTAAAACTAAGTGGTAAATCT	cme
CGGCATCGTCAGTGGAATT	CGGTTCCGAAAAAGATGGAA	cmr
TGGCCGCAGCAGAAAGA	CCGTTTTATGCACCCAGGAA	dha
GCTTTATTTCAGGAGGCGGAAT	TTTTAAATGCCACAGCACAGAATC	ereB
TCGACATGCGTGACGAGATT	CCGCGAATTAGGCCAGAA	erm30
TCGTGGGCCAGGTGAAGA	TTCCCCTTGCCGGTGAA	erm30
GCGCGTTGACGACGATTT	TGGTCATACTCGACGGCTAGAAC	erm34
TTGAAAACGATGTTGCATTAAGTCA	TCTATAATCACAACTAACCACTTGAACGT	erm35
GGCGGACCGACTTGCAT	TCTGCGTTGACGACGGTTAC	erm36
CTCTCAGAGGCTCATAGACGAAGA	TTTACGAAGTTACCAGACGTTTGTTTA	ermC
GGACTCGGCAATGGTCAGAA	CCCCGAAACGCAATATAATGTT	ermJ,D
GTTTGATATTGGCATTGTCAGAGAAA	ACCATTGCCGAGTCCACTTT	ermJ,D,K
GAGCCGCAAGCCCCTTT	GTGTTTCATTTGACGCGGAGTAA	ermK
TTTCAGCTCAGCACTTTGGTCAT	AAGGCATCTTTTTCAGCCAGTTC	fab
CGAGCAGTTCCTGCCAAAG	CCCAGTCATCCGGTTCATAATC	folA
TCACTGTAACTAATGAAGCATTAGACCAT	CCATCTGGATCTGTAAAGTAAAGAGATC	fosB
CCGGACTAGAGCTTCATGTAAGC	CCCACGCGGTACTCTTGTAAA	imiR
TTCAGATGCAATGGCGTTTG	ATAATCGGGAACATAATGAGCATAACTAC	lmrA
TGGTCAATATAACAGATGTAAACCAGATTT	CACCCCAGCCACCATCAA	lnuC
TCTGGCGTTAGCTTCACCAGTAC	GTGCAAAGGCTGGATCGAA	marA
GCGGCGTACTGGTGAAGCTA	TGCCCTGGTCGTTGATGA	marA
CCTAACGGGCGTGACTTCA	TTCACCTGTTTCAAGGGTCAAA	mdtA
ATCGGTCGCTCTTCGTTCAC	ATAAATAGGATCGAGCTGCTGGAT	mepA
TCACGACCGCCGATACCT	GCCCAAGCTCATTGACAGTCA	mphA
CTGACGCGCTCCGTGTT	GGTGGTGCATGGCGATCT	mphA
TGATGACCCTGCCATCGA	TTCGCGAGCCCCTCTTC	mphA
GAATCACTTGTCCGCAGTTTGTT	CGTACACAACGGTTTCGTCAGA	msrC
TCAGACCGGATCGGTTGTC	CCTATTTTTTGGAGTCTTCTCTCTAATGTT	msrC

Table A5 contiued

GCAAGAGCCAAGCTGCAAA	TCCGCAGCCCTTTCCAA	msrD
TAGTAGGCAAGCTCGGTGTTGA	CCTGTGCTATTTTAAGCCTTGTTTCT	msrD
GGACGGGAAGATGGTCCAA	CGTAGCGTTCCGGTTCGAT	mtrC
CGGAGTCCATCGACCATTTG	ATCGTCGGCAAGGAGAATCA	mtrC
TGCGCGTAGTCGTTCATCTC	CGTTCCAATTTCCTGATGATTG	mtrD
CCGCCAAGCCGATATAGACA	GGCCGGGTTGCCAAA	mtrD
GGTCGGCACGCTCTTGTC	TGAAGAATTTGCGCACCACTAC	mtrE
CGATGTGTCGTTTTGGAAGGT	CCTGCACCATGATTCCTCAATA	mtrE
AGCGTCAATGGCACCTTCA	AATCGAGCTGGCTGGAGTTG	mtrE
AATTTTGCCGATTATTGCTGAAA	GATTGTCATCATTCGTTTATCACCAA	multidrug
TGCGCCAAGATAGGGCATA	GTCGTGAATTCGGCAGGTTTA	nimE
GGGAGAGTTGCCGATGTTGTA	AGCCACTCGTTAAAGGGCAAT	nisB
CCCGGAGTCGATGTTCGA	GCCGAAGACGTACACGAACAG	oleC
CCGGTGCCATTGGTTTAGA	AAAATAGCCGCCCCAAGATT	pbp
GGCGAACTTCTAATTAATCCTATCCA	CGCCGATGACATTCTTCTTATCTT	Pbp5
CTTTTCTCTAACCGTACATTATCTACGATAAA	AGAACGTAGCGACTGATAAAATGCT	Protein B
TTTAGGCAGCCTCGCTTCA	CCGAATCCAAATAAAACCCAATAA	protein D
CAATAATAACCGAAATAATAGGGACAAGTT	AATAAGTGTTCCTAGTGTTGGCCATAG	qac
CATCGTGCTTGTGGCAGCTA	TGAACGCCCAGAAGTCTAGTTTT	qac
GTGGCAGCTATCGCTTGGAT	CCAACGAACGCCCACAA	qac
TCGCAACATCCGCATTAAAA	ATGGATTTCAGAACCAGAGAAAGAAA	qac
CCCCTTCCGCCGTTGT	CGACCAGACTGCATAAGCAACA	qacEdelta1
GCGGGTGTGGTCACTACGAT	AGCGTTGGGCCGATATACTG	rarD
TGACGCATCGCGTGATCT	AAATTTTCTGTGGCGTCTGAATC	rarD
CACTACCGCTTCCGCACTTAA	TGAAAAAACGGGAAAAGTCCAT	sdeAB
CAGAATCTTCCTGAAAAGTTTGATGAA	CGCAGACACGCCGAATC	spcN
AAAAGTTCGATGAAACACGCCTAT	TCCAGTGGTAGTCCCCGAATC	spcN
GCAAGAGGTATTTGCTCAACAAGA	CAGGGTCACCCTCATAAAGAAAA	speA
TTTCAGCAAGTGGATCATGTTAAAAT	CCAAGCTGTTTCCACTGTTTTTC	spec-aph
CAGCGCTATGCGCTCAAG	ATCCCGCTGCGCTGAGT	sul1
GCGATTCGCAAGGAAAGTGA	CACATGGGCCATTTTTTCATC	sulA
CAGGCTCGTAAATTGATAGCAGAAG	CTTTCCTTGCGAATCGCTTT	sulA
CACGGCTTCGGCTCATGT	TGCCATCCTGTGACTAGCTACGT	sulA

Table A5 contiued

ACCCCATGACGTACCTGTAGAGA	CAACCCACACTGGCTACCAGTT	tet35
GAGAACGTTGAAAAGGTGGTGAA	AACCAAGCCTGGATCAGTCTCA	tet37
CATATCGCAATACATGCGAAAAA	AAAGCCGCGGTAAATAGCAA	tetC
ACTGGTAAGGTAAACGCCATTGTC	ATGCATAAACCAGCCATTGAGTAAG	tetC
ATGAGTTCGGCCAGAATTTCC	GGTTGTGCGCGAAATGATT	tetR
CGCGATAGACGCCTTCGA	TCCTGACAACGAGCCTCCTT	tetR
CGCGATGGAGCAAAAGTACAT	AGTGAAAAACCTTGTTGGCATAAAA	tetR
GTGGCAAAGCAACGGATTG	TGCGGGCTTGCAAAACTATC	tetU
AACAGCGGGTTAAGTGTGCAA	ATGGTATCATTCAGTTTTCCGACAAT	tetU
GTGGCAAAGCAACGGATTG	AATGCGGGCTTGCAAAACT	tetU
CATCATCGGACGGACAGAATT	GTCGGAGATGTGGGTGTAGAAAGT	Tn21
GGGCGGGTCGATTGAAA	GTGGGCGGGATCTGCTT	Tn22
GAAACCGATGCTACAATATCCAATTT	CAGCACCGTTTGCAGTGTAAG	Tn23
GCCGCACTGTCGATTTTTATC	GCGGGATCTGCCACTTCTT	Tn24
CCGATCACGGAAAGCTCAAG	GGCTCGCATGACTTCGAATC	Tn25
ACGCCAATGCCAAACGATT	GTCACGGCGCAGCTTGA	ttgA
TCGCCCTGGATGTACACCTT	ACCATTGCCGACATCAACAAC	ttgB
ACAGGGATTGGCTATGAACCAT	TGACTGGCGATGATTTGACTATG	vanC
CCTGCCACAATCGATCGTT	CGGCTTCATTCGGCTTGATA	vanC
AAATCAATACTATGCCGGGCTTT	CCGACCGCTGCCATCA	vanC
CACACGCATTTTTTCCCATCTAG	CAGCCAACAGATCATCAAAACAA	vanC-3
TGAACAATTTTACAGGTTGGATACATCT	TCCCGTTATGAAGCTCCACTATTT	vanS
GGAAAAAGCAACTCCATCTCTTGA	TCCTGGCATAACAGTAACATTCTGA	vatB
TTGGGAAAAAGCAACTCCATCT	CAATCCACACATCATTTCCAACA	vatB
CGGAAATTGGGAACGATGTT	GCAATAATAGCCCCGTTTCCTA	vatC
TAAAAGAGAATAAGGCGCAAGGA	TGTTTAGTAGCATGTTGCATTTTCC	vgaB
GAATGATTAAGCCCCCTTCAAAA	ATTCGTGTTTCCAACGATTTCG	vgaB
AGGGAGGGTATCCATGCAGAT	ACCAAATGCGCCCGTTT	vgb
CCACGATGGCTGCCTTTG	GGCCATGCAGGACGGATAT	vgb
CGATGTTTGGATTGGACGAGAT	GCTGCAATAATAGCCCCGTTT	vgbB
CAGCCGGATTCTGGTCCTT	TACGATCTCCATTCAATTGGGTAAA	vgbB
CCGTTGCAAGAAGATTATAGAAAAAA	CAAGCATAAGACCGCATAAATGAT	yyaR

Table A6. Quantitative PCR primers used in validation of the antibiotic resistance primer set

Target Gene	Forward Sequence (5'-3')	Reverse Sequence (5'-3')	Size	Use	Annealing Temp	Reference
blaSHV	TGCTTGGCCCGAATAACAA	GCGTATCCCGCAGATAAATCA	N/A	qPCR	60	Hammond (*19*)
cfiA	GTCGCAGTTATGGCACAGAA	TCATTGATCGGTGTGTCCAG	N/A	qPCR	55	Pumbwe (*20*)
ermA	TATCTTATCGTTGAGAAGGGATT	CTACACTTGGCTTAGGATGAAA	138	qPCR	60	Diaz (*21*)
ermB	GATACCGTTTACGAAATTGG	GAATCGAGACTTGAGTGTGC	364	qPCR	58	Chen (*22*)
mexE	CCAGGACCAGCACGAACTTCTTGC	CGACAACGCCAAGGGCGAGTTCACC	114	qPCR	60	Dumas (*23*)
sul(I)	CGCACCGGAAACATCGCTGCAC	TGAAGTTCCGCCGCAAGGCTCG	163	qPCR	65	Pei (*24*)
tetA	GATATTCTGAGCACTGTCGC	CTGCCTGGACAACATTGCTT	950	PCR	55	Cabrera (*25*)
tetB	CTAATCTAGACATCATTAATTCC	TTTGAAGCTAAATCTTCTTTAT	1,397	PCR	50	Furushita (*26*)
tetC	ATGAAATCTAACAATGCGC	TCAGGTCGAGGTGGCCCGG	1,191	PCR	50	Furushita (*26*)
tetD	GAATGCCTGCACCTTTCTGATG	GGCAATAAATCCGGCGAAAA	346	qPCR	62	Fan (*27*)
tetG	GCTCGGTGGTATCTCTGCTC	AGCAACAGAATCGGGAACAC	468	qPCR	62	Fan (*27*)
tetH	GTGATGTGACTCCCGCTAAAAAT	CCAGAACCGCCAAAGACATACC	407	qPCR	62	Fan (*27*)
tetM	GAGGTCCGTCTGAACTTTGCG	AGAAAGGATTTGGCGGCACT	900	PCR	54	Zhang, 2008(*28*)
tetO	TTGTTTTGGGGCTATTGGAG	TATATGACTTTTGCAAGCTG	2,037	PCR	60	Patterson,2007(*29*)
tetS	CATAGACAAGCCGTTGACC	ATGTTTTTGGAACGCCAGAG	667	PCR	55	Ng, 2001(*30*)
tetW	TTGGGGCTGTAAAGGGAGGAC	CTTTACATTACCTTCTGA	1,949	PCR	52.5	Patterson (*29*)

References for Table A6.

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APPENDIX B. SUPPORTING INFORMATION FOR CHAPTER 3: SWINE MICROBIAL COMMUNITIES, SUBDIVIDED BY INTESTINAL LOCATIONS AND ANTIBIOTICS

Figure B1. Total assigned reads from metagenomes from lumen samples, averaged by intestinal location and treatment.

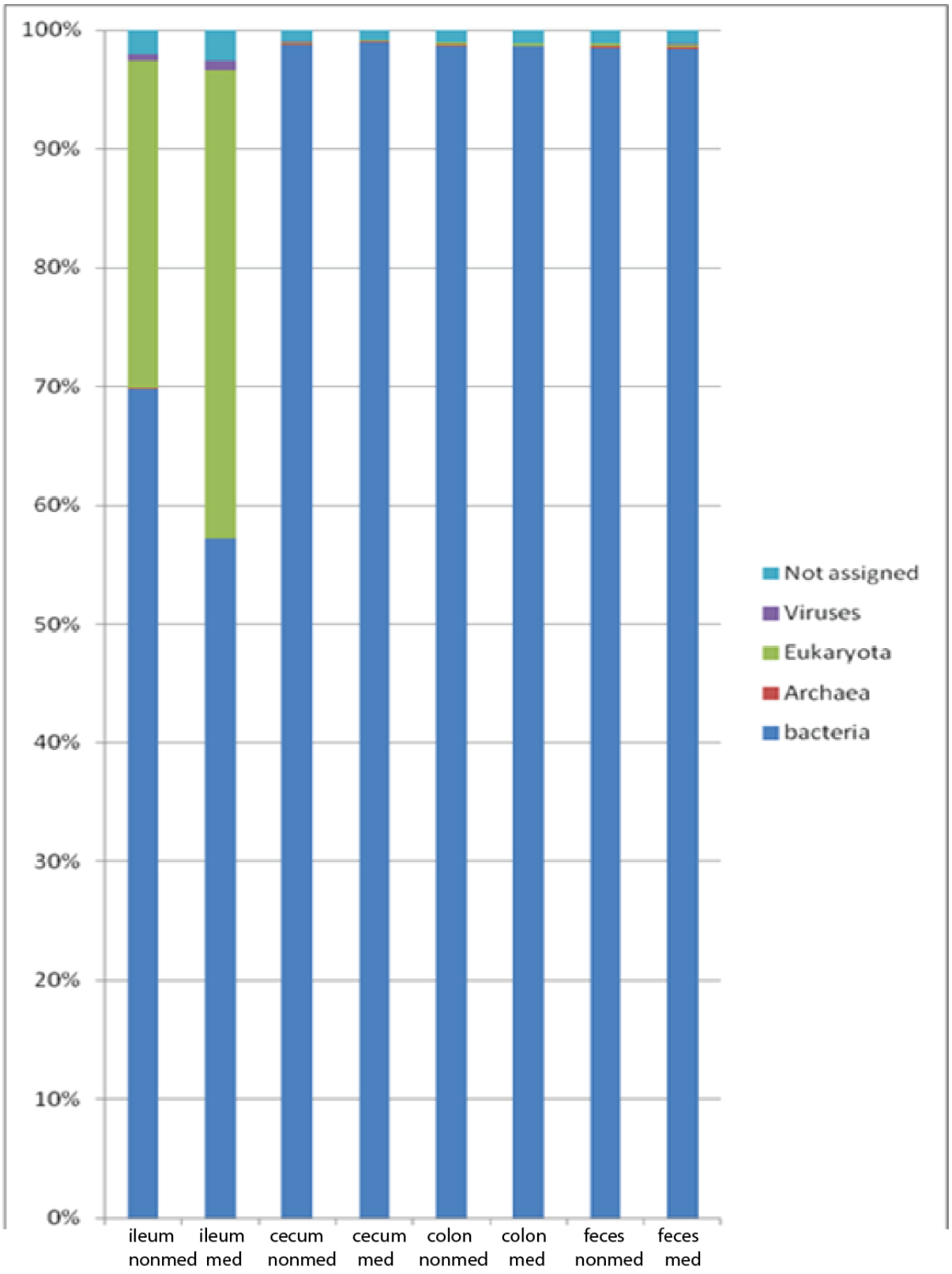

Figure B2. Stress Response assignments among the locations (non-medicated). Comparison of the number of SEED subsystem reads revealed differences among gut locations. Thirteen subsystems were significantly differently represented among the four gut locations.

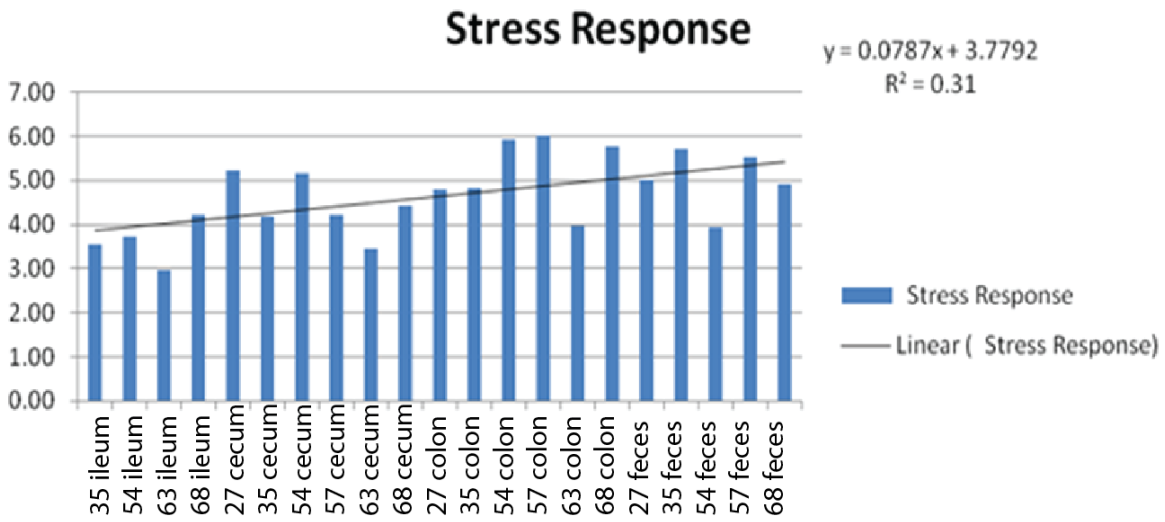

Table B1. Most abundant bacteria from the intestinal samples of swine fed ASP250 and their nonmedicated counterparts. Phylotype summary table includes top genera, normalized and averaged by treatment and intestinal site. non=nonmedicated, med=medicated

Genera	Ileum lumen non	leum lumen med	Cecum lumen non	Cecum lumen med	Colon lumen non	Colon lumen med	Feces lumen non	Feces lumen med	leum Tissue non	I leum Tissue med	Cecum Tissue non	Cecum Tissue med	Colon Tissue non	Colon Tissue med
Prevotella	0	0	971	1047	470	601	443	512	132	208	784	1049	550	956
Anaerobacter	851	1079	82	174	59	178	135	415	348	444	38	71	41	65
Turicibacter	633	310	35	18	29	10	38	15	440	341	28	16	26	8
Coprococcus	0	0	126	211	162	145	77	123	9	35	109	202	112	151
Papillibacter	0	0	37	78	109	219	209	158	5	33	42	62	94	82
Treponema	0	0	116	31	220	18	195	71	10	20	103	36	228	63
Oscillibacter	0	0	59	41	109	188	112	124	3	13	42	46	85	109
Roseburia	0	0	131	65	46	53	14	17	5	24	217	112	35	75
Bacteroides	0	0	48	69	132	146	49	40	5	17	61	85	57	77
Lactobacillus	74	153	13	48	17	29	23	18	54	148	15	91	19	29
Anaerovibrio	0	0	135	50	89	82	8	18	13	12	181	101	69	55
Escherichia	3	351	0	22	0	27	1	13	7	246	0	31	0	66
Clostridium	49	39	11	9	23	19	12	13	69	213	6	7	5	6
Succinivibrio	0	0	143	20	60	1	59	15	11	7	153	31	73	59
Streptococcus	54	0	21	0	75	0	65	0	154	21	20	1	87	1
Parabacteroides	0	0	59	56	37	43	26	52	7	14	67	63	47	66
Sporacetigenium	52	37	3	8	3	4	6	6	43	33	2	4	2	2
Ruminococcus	0	0	39	29	28	18	12	11	2	5	18	10	23	14
Butyricicoccus	0	0	5	9	18	12	29	10	2	7	9	11	23	16
Parasporobacterium	0	0	16	2	26	1	17	1	1	5	24	7	29	3
Hallella	0	0	10	16	6	22	6	8	4	5	19	19	13	18
Phascolarctobacterium	0	0	15	12	11	24	6	6	1	1	14	10	11	16
Tannerella	0	0	5	18	5	14	3	26	1	2	6	26	4	18

Pseudomonas	0	0	0	0	0	0	0	0	27	29	1	0	1	0
Anaerostipes	0	0	7	6	9	17	4	1	0	2	6	3	7	10
Anaeroplasma	0	0	2	17	0	3	0	9	1	2	2	22	1	24
Faecalibacterium	0	0	12	5	8	2	2	1	1	3	15	11	7	6
Blautia	0	0	4	4	14	5	10	3	1	4	4	4	13	4
Campylobacter	0	0	0	0	0	0	0	0	25	1	8	1	2	1
Dorea	0	0	6	11	4	10	1	10	0	1	4	5	3	6
Barnesiella	0	0	3	6	4	6	5	8	1	2	3	8	5	9
Bradyrhizobium	0	0	0	0	0	0	0	0	18	10	0	0	0	0
Anaerotruncus	0	0	3	1	14	6	4	3	0	1	4	0	7	3
Fibrobacter	0	0	16	0	5	1	6	3	6	0	11	0	4	2
TM7_genera_ incertae_sedis	0	0	6	1	11	1	7	0	0	1	4	1	10	1
Salsuginibacillus	0	0	0	9	0	11	0	6	0	2	0	5	0	11
Hespellia	0	0	6	4	5	3	2	2	0	1	4	4	4	4
Subdoligranulum	0	0	4	2	5	3	4	3	1	1	4	4	5	3
Spirochaeta	0	0	2	2	3	1	5	6	0	1	3	2	7	5
Mitsuokella	0	0	4	0	5	2	5	0	1	1	2	2	10	4
Alistipes	0	0	4	1	4	7	2	0	0	2	4	3	5	5
Eubacterium	0	0	0	1	4	5	3	2	0	1	1	1	5	3
Sutterella	0	0	4	1	0	0	0	1	1	1	9	4	2	3
Lachnobacterium	0	0	0	4	0	10	0	3	0	0	0	2	0	5
Acetivibrio	0	0	2	1	5	2	3	3	0	1	2	0	3	1
Peptostreptococcus	0	0	1	1	3	2	1	3	0	1	1	2	3	3
Catenibacterium	0	0	1	3	1	4	1	0	0	1	2	2	1	2
Eggerthella	0	0	2	0	5	2	1	0	0	0	2	1	3	1
Peptococcus	0	0	0	1	2	2	1	0	0	1	2	3	3	1
Butyricimonas	0	0	1	1	2	1	3	2	0	0	1	2	1	3
Pyramidobacter	0	0	2	1	2	1	1	0	0	0	2	1	3	2

Coprobacillus	0	0	1	2	0	6	0	3	0	0	0	0	1	3
Paraprevotella	0	0	0	6	0	1	0	2	0	1	0	4	0	2
Subdivision5_ genera_incertae_sedis	0	0	0	0	1	0	2	4	0	0	1	0	2	2
Lentisphaera	0	0	0	0	2	0	4	1	0	0	0	0	3	1
Oribacterium	0	0	3	1	1	1	0	2	0	0	1	0	1	1
Anaerofilum	0	0	1	0	3	0	1	1	0	0	0	0	1	1
Pseudobutyrivibrio	0	0	2	1	1	0	1	0	0	0	2	0	0	0
Enterobacter	0	1	0	0	0	0	0	0	3	1	0	0	0	0
Akkermansia	0	0	0	0	0	1	0	1	0	0	3	0	0	1
Weissella	0	0	0	0	0	0	0	0	4	0	0	0	0	0
Acetanaerobacterium	0	0	0	0	0	1	0	3	0	0	0	0	0	1
Jonquetella	0	0	0	0	1	0	3	0	0	0	0	0	2	0
Mogibacterium	0	0	0	0	1	0	1	2	0	1	0	0	1	1
Robinsoniella	0	0	1	1	0	1	0	0	0	0	0	1	0	1
Rhodopirellula	0	0	0	0	2	0	1	0	0	0	0	0	1	0
Allobaculum	0	0	0	1	1	1	0	1	0	0	0	1	0	1
Helicobacter	0	0	0	0	0	0	0	0	0	0	5	0	0	0
Anaerovorax	0	0	1	0	1	0	1	1	0	0	0	0	1	0
Cronobacter	0	0	0	0	0	0	0	0	3	0	0	0	0	0
Actinobacillus	1	0	0	0	0	0	0	0	3	0	0	0	0	0
Anaerosporobacter	0	0	1	0	0	0	0	0	0	0	1	0	0	0
Xylanibacter	0	0	1	0	0	0	0	1	0	0	0	0	0	2
Acholeplasma	0	0	0	0	0	0	0	0	0	1	0	1	0	1
Erysipelothrix	0	0	0	0	0	1	0	1	0	0	0	0	0	0
Megasphaera	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Solobacterium	0	0	0	0	1	1	0	0	0	0	0	0	0	0

APPENDIX C. ANTIBIOTICS IN FEED INDUCE PROPHAGES IN SWINE FECAL MICROBIOMES

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This work has been published elsewhere.

Allen HK, Looft T, Bayles DO, Humphrey S, Levine UY, Alt D et al (2011). Antibiotics in Feed Induce Prophages in Swine Fecal Microbiomes. *mBio* 2. I designed this experiment with the listed coauthors. Additionally, I collected samples, extracted DNA, performed the 16S rRNA gene PCR reactions, analyzed 16S rRNA data, and wrote the methods, results and discussions sections that relate to the phylotype analysis.

Abstract

Antibiotics are a cost-effective tool for improving feed efficiency and preventing disease in agricultural animals, but the full scope of their collateral effects is not understood. Antibiotics have been shown to mediate gene transfer by inducing prophages in certain bacterial strains; therefore, one collateral effect could be prophage induction in the gut microbiome at large. Here we used metagenomics to evaluate the effect of two antibiotics in feed (carbadox and ASP250 [chlortetracycline, sulfamethazine, and penicillin]) on swine intestinal phage metagenomes (viromes). We also monitored the bacterial communities using 16S rRNA gene sequencing. ASP250, but not carbadox, caused significant population shifts in both the phage and bacterial communities. Antibiotic resistance genes, such as multidrug resistance efflux pumps, were identified in the viromes, but in-feed antibiotics caused no significant changes in their abundance. The abundance of phage integrase-encoding genes was significantly increased in the viromes of medicated swine over that in the viromes of nonmedicated swine, demonstrating the induction of prophages with antibiotic treatment. Phage-bacterium population dynamics were also examined. We observed a decrease in the relative abundance of *Streptococcus* bacteria (prey) when *Streptococcus* phages (predators) were abundant, supporting the “kill-the-winner” ecological model of population dynamics in the swine fecal microbiome. The data show that gut ecosystem dynamics are influenced by phages and that prophage induction is a collateral effect of in-feed antibiotics.

Importance

This study advances our knowledge of the collateral effects of in-feed antibiotics at a time in which the widespread use of “growth-promoting” antibiotics in agriculture is under

scrutiny. Using comparative metagenomics, we show that prophages are induced by in-feed antibiotics in swine fecal microbiomes and that antibiotic resistance genes were detected in most viromes. This suggests that in-feed antibiotics are contributing to phage-mediated gene transfer, potentially of antibiotic resistance genes, in the swine gut. Additionally, the so-called “kill-the-winner” model of phage-bacterium population dynamics has been shown in aquatic ecosystems but met with conflicting evidence in gut ecosystems. The data support the idea that swine fecal *Streptococcus* bacteria and their phages follow the kill-the-winner model. Understanding the role of phages in gut microbial ecology is an essential component of the antibiotic resistance problem and of developing potential mitigation strategies.

Introduction

The Infectious Diseases Society of America is among the organizations that have recommended that the U.S. government limit the use of antibiotics in agriculture (1, 2). The European Union has banned the use of all agricultural antibiotics that are used for growth promotion (3, 4). However, various factors, notably the cost-effectiveness of antibiotic use for performance benefits in modern, conventional agricultural practices (5), have forestalled similar measures in the United States. Partly driving the ongoing debate on the prudence of the widespread use of antibiotics to improve feed efficiency in agricultural animals (6, 7) is a growing body of research on the collateral effects of these antibiotics, ranging from an increased abundance of antibiotic resistance genes in pigs fed antibiotics (T. P. Looft, T. Johnson, H. K. Allen, D. O. Bayles, D. P. Alt, R. D. Stedtfeld, W.-J. Sul, T. M. Stedtfeld, B. Chai, S. A. Hashsham, J. M. Tiedje, and T. B. Stanton, submitted for publication) to the

modulation of bacterial gene expression by subinhibitory concentrations of antibiotics (8). Another collateral effect could be the induction of gene transfer among bacteria.

Horizontal gene transfer is a mechanism by which bacteria exchange genetic material and is known to occur among gut bacteria (9). One type of horizontal gene transfer is mediated by phages. Some antibiotics are known to affect prophage-mediated gene transfer in certain bacteria *in vitro*. The virus-like gene transfer agent VSH-1 is induced by carbadox in the swine pathogen *Brachyspira hyodysenteriae* and transfers antibiotic resistance genes (10, 11). Also, beta-lactam antibiotics and fluoroquinolones induce prophages in *Staphylococcus aureus*, some of which package pathogenicity islands and therefore transfer virulence traits (12, 13). However, the *in vivo* effects of antibiotics on phages in the gut are unknown.

Phage diversity and function have been studied in water, soil, and animal-associated environments, but only a small fraction of phages have been characterized (14). Metagenomic analyses enable the study of phages without isolating them (15). The phage metagenome, or virome, is the sequenced assemblage of the total phages of a microbial community. Recently, phage metagenomic analyses have launched studies comparing phage ecology between environments. In such studies, phage diversity and functions can be elucidated despite a limited understanding of specific phages in a given community, such as the demonstrated increase in phage diversity in the lungs of cystic fibrosis patients (16).

Our goal was to examine the fecal viromes over time in swine that were fed the common antibiotics carbadox and ASP250. The viromes were compared to those of nonmedicated swine and to the corresponding bacterial communities. The data show that in-feed antibiotics induce prophages in the swine intestine and cause significant shifts in both

phage and bacterial community structures. Additionally, analysis of the relative abundance of *Firmicutes* bacteria and phages, specifically the *Streptococcus* spp., unexpectedly revealed that the predator-prey population dynamics model called “kill-the-winner” might apply to the swine microbiome.

Results

Diverse phages in swine feces.

Phages in each sample were visualized by electron microscopy, revealing members of the *Siphoviridae*, *Myoviridae*, and *Podoviridae* phage families based on morphology (Fig. C1a to C1d). Several enveloped viruses were also seen (Fig. C1e). Frequently, many phages could be visualized in a field of view (Fig. C1f). No bacterial cells were seen by electron microscopy. Lack of bacterial cell contamination of phage preparations was further confirmed by the inability to amplify the 16S rRNA gene from virome samples.

ASP250 causes shifts in phage membership.

Viromes were analyzed by MG-RAST (17) and GAAS (18) to classify the sequences based on their putative origins. Out of nearly 1 million phage metagenomic sequence reads, 44% of the sequences (average across viromes) had no hits in the databases according to MG-RAST (Fig. C2a). Few virome sequences were solely assignable to phage or virus origins, while considerably more virome sequences were attributed to bacterial origins (Fig. C2a). GAAS was used to infer phage taxonomy based on the best match according to BLASTx. Only phages that occurred at ≥0.1% abundance in at least one virome were analyzed. Approximately 90% of assignable phages were attributed to phages of canonical

gut bacteria, and the vast majority of assignments were to bacteria of the *Firmicutes* phylum (Fig. C2b; Supplemental Fig. C1). ASP250 but not carbadox caused considerable shifts in the phage community compared to control and pre-ASP250-fed animals ($R = 0.72$, $P < 0.10$).

Phage community shifts are paralleled in the bacterial community.

We compared phage diversity to bacterial diversity by amplifying and analyzing 16S rRNA gene sequences from the swine fecal samples. Sequencing the V1-to-V3 region of the 16S rRNA gene of fecal DNAs yielded 1,077,133 sequences from 86 bar-coded samples. All samples were dominated by four phyla: *Bacteroidetes*, *Firmicutes*, *Spirochaetes*, and *Proteobacteria* (Fig. C3a), and the identified genera are typical for a mammalian gut environment (Fig. C3b). Specific genera detected at lower levels ($P < 0.01$) in ASP250-treated animals than in corresponding nonmedicated animals (day 0 and nonmedicated control animals) were *Coprococcus*, *Succinivibrio*, *Streptococcus*, *Treponema*, and *Turicibacter* spp. (Fig. C3b; Supplemental Table C1). Additionally, *Escherichia* sp. increased with in-feed ASP250, although the overall abundance of this genus remained relatively low (Fig. C3b).

To visualize changes in bacterial diversity, a principal component analysis of operational taxonomic units (OTUs) was performed. The bacterial community shifted in animals medicated with ASP250 compared to that in nonmedicated animals (both ASP250 on day 0 and all corresponding control animals; $P < 0.01$, $R = 0.43$; Fig. C3c), mirroring the results shown for the phage communities. The shift was driven in part by total bacterial OTU diversity decreases in the day 14 ASP250-treated samples compared to the control animals

(see Table S2 in the supplemental material). No significant changes in bacterial diversity were detected with in-feed carbadox treatment (Table S2).

Kill-the-winner population dynamics are displayed in the swine fecal microbiome.

Predator-prey relationships between swine fecal phages and bacteria were examined. *Firmicutes* bacteria and their associated phages were analyzed because they made up a large proportion of the assignable virome data (Fig. C2b). Even at the phylum level, the dynamic relationship between phages and bacteria was apparent in the nonmedicated swine viromes over time (Fig. C4a). A regression analysis of all viromes revealed that when the *Firmicutes* phages were present in relatively high abundance, the abundance of *Firmicutes* bacteria tended to be lower ($r^2 = 0.21$, $P < 0.1$; Fig. 4b). Of the *Firmicutes*, the *Streptococcus* genus was analyzed because its members were relatively abundant in both the phage and bacterial data sets. As with the *Firmicutes*, *Streptococcus* bacteria decreased as *Streptococcus* phages increased ($r^2 = 0.23$, $P < 0.1$; Fig. 4c and 4d). Only three other genera were present in both the phage and bacterial relative abundance data, and they all showed the same trend to various degrees (*Escherichia*, $r^2 = 0.45$; *Lactobacillus*, $r^2 = 0.13$; *Clostridium*, $r^2 = 0.03$). These data suggest that the kill-the-winner model of population dynamics applies to swine gut microbial communities.

Functional analysis of swine viromes reveals fitness genes.

Putative functions of coding sequences were collated by CAMERA (19) and analyzed. Clusters of orthologous groups (COGs) revealed an emphasis on DNA replication and transcription in the viromes (Supplemental Fig. C3). Multivariate analyses, in addition to

statistical analyses of the COG assignments using ShotgunFunctionalizeR (20), revealed no significant patterns of COGs based on time or treatment.

We hypothesized that antibiotic resistance would be one type of bacterial fitness trait encoded by phages; therefore, we searched for antibiotic resistance functions in all viromes. Sequences encoding resistance to antibiotics and toxins were annotated by MG-RAST (see Table S3 in the supplemental material) (17), and further details were acquired by comparing the viromes to the antibiotic resistance gene database (ARDB [21]) (Table 1). According to the ARDB, most viromes harbored few antibiotic resistance genes (107 genes out of 1,036,084 total reads [0.01%]), and two viromes had no detectable resistance genes. Eight resistance genes occurred more than twice across all viromes, and most of these encoded efflux pumps (Table 1). Normalized resistance gene frequencies were analyzed in multivariate analyses but showed no discernable pattern based on time or treatment.

In-feed antibiotics induce prophages in the swine gut.

A potential indicator of the effect of antibiotics on prophages could be genes encoding phage integrases (S. Casjens, personal communication). Genes annotated as encoding an integrase were enumerated by MG-RAST and normalized by the total number of reads per virome. To gain statistical power, both carbadox- and ASP250-treated swine viromes were grouped as “medicated.” The viromes of medicated pigs ($n = 5$) harbored more integrase genes than did the viromes of nonmedicated pigs ($n = 10$, $P < 0.01$) (Fig. C5). This indicates that in-feed antibiotics induced prophages from gut bacteria, with a surge in the abundance of integrase-encoding genes in the ASP250-treated swine virome at day 8.

Discussion

This is the first report of the effect of antibiotics on total phage diversity in a microbial community. The results show that a collateral effect of antibiotic treatment is increased abundance of phage integrase-encoding genes, reflecting the induction of prophages from gut bacteria. Integrases are an appropriate marker for prophage induction because they are required for temperate phages transitioning from lysis to lysogeny (22). Integrases are also associated with pathogenicity islands, which are often mobilized by prophages (23). A greater abundance of integrases with antibiotic treatment, therefore, indicates that antibiotics are inducing phage-mediated bacterial lysis in the gut. Integrase abundance increased regardless of the type of in-feed antibiotic; further research is required to determine the specificity of perturbations that result in increased integrase abundance.

Additional consequences of in-feed antibiotic-mediated phage induction could be increased abundance of bacterial fitness or virulence genes, such as those encoding antibiotic resistance. Various homologues of antibiotic resistance genes were detected in the swine viromes at a frequency corresponding to approximately 1/50 of the frequency of antibiotic resistance genes in an *Escherichia coli* genome. Particularly in the context of no detection of 16S rRNA genes by PCR in the viromes, the apparent low number of resistance-encoding virome reads seems surprisingly frequent. Resistance genes were identified slightly more frequently in human fecal viromes (0.1% [24]). With selective pressure, any phage-transferred resistance genes could accelerate the evolution of resistance in the gut microbiome. Despite the potential relevance of the transduction of resistance genes in an antibiotic-containing environment, the swine viromes provided no evidence of a treatment effect. Increased sequencing depth may be required to detect differences among the viromes,

such as the effect of generalized transduction on a virome. Additionally, transcriptomic analyses would demonstrate which phage genes have altered expression as a result of antibiotic treatment, revealing those genes important for fitness in an antibiotic environment.

Phages have been shown to play an important role in ecosystem dynamics (25), and one dynamic is the relationship between phages and bacteria. A widely investigated model for this relationship is called kill-the-winner (26). This model predicts that an increase in a given bacterial host population (winning prey) results in an increase in its phages (predators) and subsequent predation of the winner. Kill-the-winner population dynamics have been supported in marine ecosystems (27), but it is unclear if the model holds true for gut ecosystems. The extended sampling (seven viromes over time) of nonmedicated animals' viromes presented a ripe data set for investigating phage-bacterial population dynamics. Overall, the nonmedicated swine viromes showed taxonomic and functional stability over time, as seen in aquatic microbiomes (27). Despite this apparent stability, examination of the relative abundances of *Streptococcus* phages compared to *Streptococcus* bacterial abundances over time revealed a dynamic process resembling kill-the-winner. Indeed, the swine viromes suggest that the kill-the-winner process might be detectable at the phylum level. This is consistent with other work that has shown kill-the-winner dynamics at the strain level in aquatic microbiomes (27) and horse feces (28). However, a fecal phage metagenomic study from pairs of twins and their mothers revealed little intravirome change across three sampling dates, and the authors refuted the model (29). Two major differences between the present study and the twin study are that we isolated phages from fresh (not frozen) feces and that we did not employ a DNA amplification step prior to sequencing. These protocol improvements were designed to reduce bias in analyses of phage diversity (30), enabling us

to view population dynamics even in complex ecosystems such as the swine microbiome. The results tentatively support kill-the-winner dynamics in swine microbiomes, but more research is required to resolve the applicability of the kill-the-winner model across mammalian gut ecosystems.

Analysis of the in-feed ASP250 viromes suggests that there is an antibiotic effect on the relative abundance of fecal phages. The only component of ASP250 known to have an effect on phage lysis is penicillin. Subinhibitory concentrations of penicillin were shown to weaken *Streptococcus* spp. such that even so-called phage-resistant strains in mixed cultures were susceptible to phage lysis by exogenous phages (31). This could account for the significant decrease in *Streptococcus* spp. with ASP250, although it provides no evidence for the concomitant increase in *Streptococcus* phages. A related phenomenon is called phage-antibiotic synergy (PAS) and has been demonstrated with diverse phages of *E. coli* in the presence of subinhibitory concentrations of various cephalosporin-type beta-lactam antibiotics (32). The result of PAS is phage induction, and it is independent of an SOS response and dependent on a filamentation phenotype resulting from certain antibiotic treatments (32). Taken together, these data suggest that penicillin is the active component of ASP250 that is affecting the phage population, perhaps by numerous and complex mechanisms in the bacterial milieu.

Analysis of the structure of the bacterial communities shows that a small, important fraction of the data is driving the shift in diversity with ASP250 treatment. The decrease in the lactic acid bacterium (LAB) *Streptococcus* is particularly intriguing and agrees with the reported decrease in the abundance of LABs with certain oral antibiotic treatments (33–35). This decrease is often accompanied by an increase in *Proteobacteria*, specifically in

Escherichia populations as shown here and elsewhere (33; Looft et al., submitted). In addition to the immediate effects on the microbiota, oral antibiotic treatment was shown to decrease the immune response in mice, even in distant locations such as the lungs (34, 35). The interaction of LABs with the gut mucosa is thought to be immunomodulatory (36), so perhaps there is a connection between the abundance of LABs and immune function. Interestingly, a recent study evaluating in-feed fumaric and formic acids showed a trend towards increased abundance of coliforms and decreased lactobacilli in plate counts (37), mirroring the effect of ASP250 on the microbiota. Fumaric acid has been demonstrated to improve weight gain despite no changes in available energy in the gut (38). Furthermore, in a study of irritable bowel syndrome, subjects with a higher body mass index than that of normal subjects had fewer lactobacilli (a type of LAB) (39). Considering that one mechanism of antibiotic-mediated growth promotion could be suppressed immune response due to decreased bacterial load (40), a decrease in immunomodulatory LABs might also decrease the energy spent on immunity and allow for increased feed efficiency.

The fecal bacterial diversity in the current study supports what has been shown previously: the swine gut is dominated by *Firmicutes* (~30%), *Bacteroidetes* (~50%), and *Proteobacteria* (~10%). However, the proportion of assignable phage sequences does not mirror this distribution, with nearly 80% of reads called from phages of bacteria of the *Firmicutes* phylum. Their inflation in our data set could result from the overrepresentation of phages of *Firmicutes* in public databases compared to those of *Bacteroidetes*, perhaps because of increased research interest due to their potential biotechnological applications (41). Additionally, phage sequences are simply lacking in the databases compared to bacterial sequences, limiting the pool of potential homologues for the swine viromes.

A relatively large proportion of assignable virome sequences were of bacterial origin despite no detectable bacterial contamination of the viromes. Phages harbor more bacterial genes than previously appreciated (42), making it reasonable that the assignable sequences of the swine viromes have 46% bacterial genes even in the absence of bacterial contamination. Also, generalized transducing phages package host bacterial DNA, contributing an unknown proportion to the counted bacterial genes.

No statistically significant effect of carbadox was detected on swine fecal phages or bacteria. Different sampling intervals and minor protocol improvements between the carbadox and ASP250 experiments (see Materials and Methods) may have affected the results. Our results suggest that 1 week following the commencement of in-feed antibiotics is an appropriate time to detect changes in the fecal microbiome.

This study provides evidence that a collateral effect of some in-feed antibiotics, such as ASP250, is the induction of prophages. Additionally, antibiotic resistance genes were detected in the phage metagenomes. Further work is required to determine the implications of prophage induction on the transfer of antibiotic resistance genes. Surprisingly, the data also support the kill-the-winner model for phage-bacterium population dynamics. Taken together, the data underscore the importance of phages in complex microbial communities.

Materials and Methods

Swine.

Three rooms that would house pigs were decontaminated prior to the beginning of the study. All animals were managed in accordance with National Animal Disease Center Animal Care and Use Committee guidelines. Three pregnant sows farrowed on site, and piglets were weaned after 14 days. Weaned pigs were divided into three groups with approximately equal representation of littermates and gender, with two groups of six pigs housed in their own clean rooms and the remaining pigs housed in a third room (Supplemental Fig. C2). All pigs were fed the same diet (TechStart 17-25; Kent Feeds, Muscatine, IA) for 1 week after weaning and until the start of their respective study, at which point six control pigs continued to receive TechStart while three groups of six experimental pigs received one of the following in-feed supplements: subtherapeutic carbadox, 10 g/ton; therapeutic carbadox, 50 g/ton; or ASP250 (chlortetracycline, 100 g/ton; sulfamethazine, 100 g/ton; penicillin, 50 g/ton). For a 4.5-kg pig, this equaled the following concentrations of antibiotic per gram of pig per day: 8.2 µg therapeutic carbadox; 1.6 µg subtherapeutic carbadox; and 16.4 µg chlortetracycline, 16.4 µg sulfamethazine, and 8.2 µg penicillin (ASP250). Note that these concentrations decreased as the pigs increased in size. Freshly voided fecal samples were collected from control and medicated animals just before treatment (day 0) and 3, 8, 14, or 28 days after continued treatment (Supplemental Fig. C2). Feces were transported on ice from the barn to the lab and were immediately processed for phage isolation and stored at −20°C for bulk DNA extraction.

Phage isolation.

The following protocol was adapted from previous reports (43–45). Approximately equal amounts of feces from each of the six animals in a treatment group were pooled to roughly 10 g. Feces were pooled because of the low biomass of phages and to avoid biasing the samples by amplifying the DNA in a later step (30). Pooled feces were blended in 50 ml SM buffer (8 mM $MgSO_4$, 100 mM NaCl, 50 mM Tris-Cl, and 0.002% [wt/vol] gelatin) in a Waring blender (Torrington, CT) for 30 s. Fecal slurries were poured through a Nitex mesh (~118-μm pore size; Wildlife Supply, Yulee, FL) into a sterile centrifuge bottle. Samples were centrifuged three times at 4°C, first at 3,000 × *g* for 10 min and then twice at 10,000 × *g* for 10 min. The supernatants were carefully transferred to sterile centrifuge bottles between spins or to 50-ml Falcon tubes following the final spin. CsCl was added to the final supernatant to a density of 1.15 g/ml.

Meanwhile, two CsCl gradients were prepared per fecal sample (44, 45). Gradients were ultracentrifuged at 37,946 × *g* for 2 h at 4°C in a Beckman SW28 rotor. A 20-gauge needle on a 1-ml syringe was used to draw off 1 ml containing concentrated virions from the interface between the 1.35- and 1.5-g/ml layers. To improve DNA yield, the needle was inserted in the middle of the 1.5-g/ml layer and the volume of virions extracted was increased to 5 ml for the six samples taken during the ASP250 experiment. Because of a brown, stringy substance suspended vertically through the gradient, all virions were gently filtered through a 0.45-μm syringe filter into an Ultracel 3K regenerated cellulose concentrator (Millipore, Billerica, MA). Samples were gently centrifuged (<3,000 × g) until the sample volume was ~1 ml.

TEM visualization of phage particles.

An aliquot of virions was washed and concentrated for transmission electron microscopy (TEM) visualization. Briefly, 50 µl of virion-containing CsCl was applied to a Microcon YM-100 filter tube (Millipore) and washed twice with 500 µl SM buffer. Virions were resuspended in 10 µl SM buffer and stored at 4°C until TEM. On the day of TEM, 10 µl of virions was mixed with 10 µl of fresh 2% phosphotungstic acid (pH 7.0) and incubated at room temperature for 3 minutes. A Formvar- and carbon-coated 200-mesh copper grid (Electron Microscopy Sciences, Hatfield, PA) was introduced into this mixture for 1 min. The excess fluid was wicked away with filter paper. Grids were viewed on a Tecnai G^2 Biotwin transmission electron microscope (FEI, Hillsboro, OR).

Phage DNA isolation.

Phage DNAs were isolated as described previously (45). Briefly, intact virions were treated with DNase to eliminate free DNA. Following DNase inactivation, virions were lysed with formamide and the DNA was precipitated. DNA was extracted by sequential SDS, cetyltrimethylammonium bromide (CTAB), and chloroform treatments.

Metagenomic sequencing and analysis.

Individual preparations of phage DNA were quantified using Quant-iT PicoGreen (Invitrogen, Carlsbad, CA). Five hundred nanograms of DNA from each preparation was used in the Rapid Library Preparation method (454 Life Sciences, Branford, CT). Libraries were created with Roche multiplex identifier (MID)-labeled adaptors. Individual libraries were pooled into one of two groups in an equimolar fashion. Pooled preparations were used

to prepare DNA beads for sequencing using a two-region picotiter plate on a Roche GS-FLX instrument using Titanium chemistry (454 Life Sciences).

The metagenome sequences were processed with the 454 Replicate Filter, extract_replicates.py script, to remove artificially replicated sequences (46). Sequences in a cluster were removed as artificial replicates if the first three bases were identical and there was greater than 90% identity over the length of the shortest sequence in a cluster (see Table S3 in the supplemental material). Dereplicated reads were uploaded to CAMERA (https://portal.camera.calit2.net [19]), and the RAMMCAP pipeline sorted the reads for encoded functions, including separation by clusters of orthologous groups (COGs). Domain taxonomy and integrases were determined by MG-RAST (http://metagenomics.anl.gov/ [17]). The dereplicated virome reads were analyzed with GAAS to obtain estimates of the viral genome sizes as well as confidence intervals of the estimates (18). PAST (47) was used to perform one-way ANOSIM and principal component analyses on the normalized (percentage of the total number of assigned reads per virome) phage relative abundance data from GAAS. ShotgunFunctionalizeR was used to make statistical comparisons of COG assignments among viromes (20). To draw statistical conclusions, the following cutoffs were indicated: $P < 0.1$, trend; $P < 0.01$, significance; $R = 0$ to 0.3, slight correlation; $R = 0.3$ to 0.5, medium correlation.

16S rRNA gene sequence analysis.

16S rRNA gene sequences were amplified from the fecal samples of individual pigs. PCR amplification of the V1-to-V3 region of bacterial 16S rRNA genes was carried out with the conserved primers 8F (5′-AGAGTTTGATCCTGGCTCAG [48]) and 518R (5′-

ATTACCGCGGCTGCTGG [49]) with sequence tags (bar codes) and sequencing primers incorporated into each PCR primer (see Table S4 in the supplemental material). PCR mixtures contained 200 μM (each) deoxyribonucleotide triphosphate, 2.0 μM (each) primer, 2.0 U Ampligold *Taq* polymerase (Applied Biosystems, Foster City, CA), 2.5 mM $MgCl_2$, 50 ng template DNA, Ampligold *Taq* buffer (Applied Biosystems), and water to 50 μl. PCRs were performed in a PTC-225 thermal cycler (MJ Research, Watertown, MA) with the following protocol: 3 min at 95°C; 21 cycles of 1 min at 95°C, 30 s at 56°C, and 45 s at 72°C; and a final elongation step for 3 min at 72°C. PCR for the 16S rRNA gene was also performed on purified phage metagenomic DNAs, but the number of cycles was increased to 40. PCR products were separated by gel electrophoresis and purified using the MinElute kit (Qiagen Inc., Valencia, CA). They were then sequenced on a 454 Genome Sequencer FLX, using the manufacturer's protocol for Titanium chemistry (Roche Diagnostics, Branford, CT). Data were processed per manufacturer's protocols, and AmpliconNoise (50) was used to reduce sequence artifacts produced during PCR and sequencing.

Phylotype analysis.

After binning the samples by bar code, phylogenetic analysis and taxonomic assignments of the 16S rRNA gene sequences were made using the Ribosomal Database Project (RDP) web tools (51). Additional phylotype comparisons and hypothesis testing were performed with the software package mothur (52). OTU abundances (97% similarity) were normalized to the total number of OTUs per sample, and these data were subjected to principal component analyses in mothur (52). PAST (47) was used to plot the data, allowing for the visualization of the relationships between samples. The estimated total diversity of

operational taxonomic units was calculated with Catchall (53). Regression analyses were performed in Excel ($P < 0.1$, trend; $P < 0.01$, significance).

Nucleotide sequence accession numbers.

All sequences (phage metagenomes and 16S rRNA gene sequences) were deposited in NCBI (BioProject PRJNA72355; Sequence Read Archive accession number SRA045429) and are additionally available through CAMERA and MG-RAST.

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Figure C1. Electron micrographs of virions isolated from swine feces. (A to D) Representative phages of the *Myoviridae* (A), *Siphoviridae* (B [no arrow] and C), and *Podoviridae* (B [arrow] and D) families. (E) An enveloped virus as seen in numerous fecal samples of young pigs. (F) Ten *Siphoviridae* (arrows) were visualized in a single field.

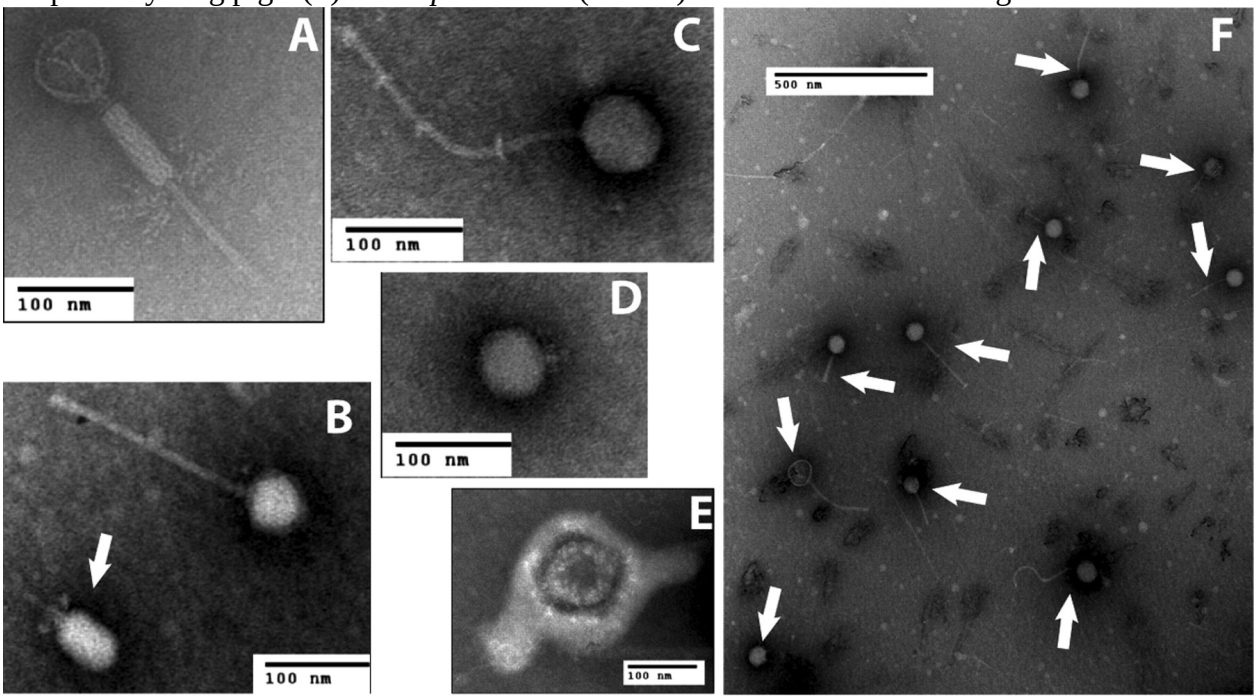


Figure C2. Community structure based on taxonomic inference of phages from swine feces. (A) Phage metagenomic sequence origins. “No hits” is the percentage of reads with no similar sequences in the database, and “unassigned or unclassified” is the percentage of reads with a database hit that has no associated taxonomic information. The values on the graph are the medians, and the values in parentheses are the ranges. (B) Genus-level phylogenetic origins of the phage-derived sequences from the ASP250 experiment. Phage taxa occurring at roughly <0.5% abundance were grouped as “Other phages.” Asterisks denote those of the *Firmicutes* phylum.

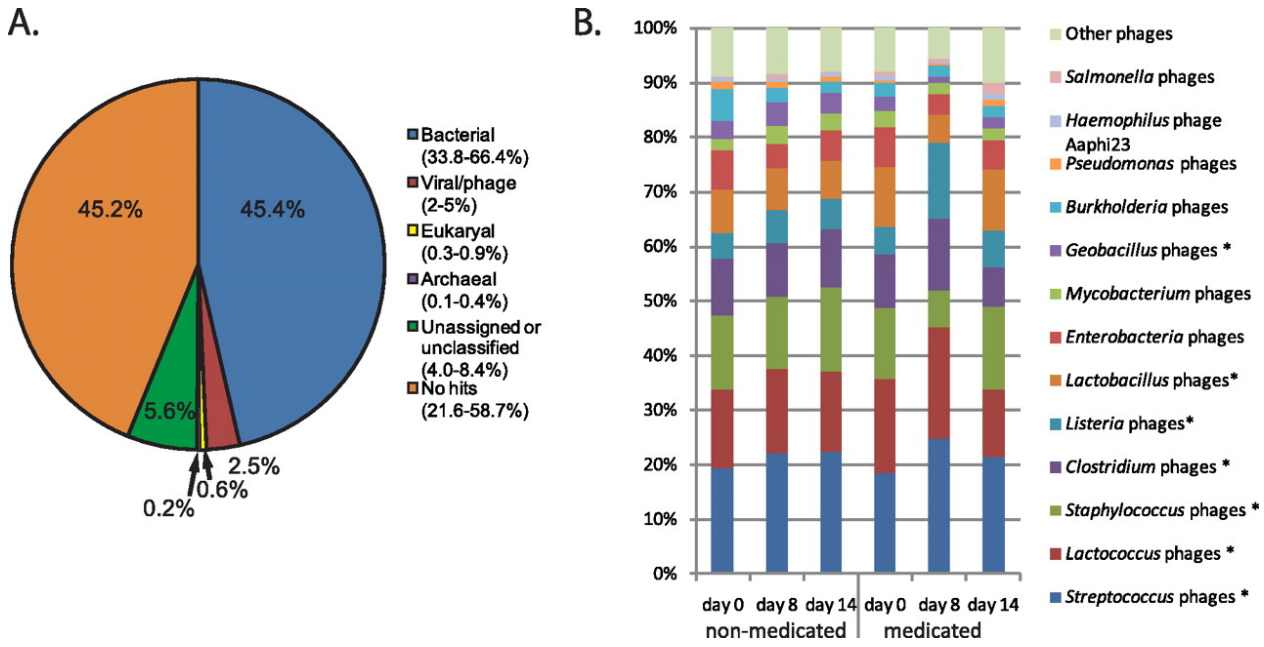


Figure C3. Community structure based on taxonomic inference of bacteria (16S rRNA sequences) from swine feces. (A) Phylum-level assignments of assignable 16S rRNA gene sequences from swine feces, averaged across all 86 individual samples. The values on the graph are the medians among treatment groups, and the values in parentheses are the ranges. (B) Average percent abundance of genus-level assignments of 16S rRNA gene sequences from the feces of six swine fed ASP250 and the corresponding nonmedicated animals. Values were normalized to the total number of assignments within a sample. Taxa occurring at roughly <0.3% abundance were grouped as "Others." Asterisks denote those of the *Firmicutes* phylum. See Table S1 in the supplemental material for the values of the means and the standard errors. (C) Principal component analysis of OTU-based bacterial 16S rRNA gene sequence abundances in individual pig samples ($P < 0.01$, $R = 0.43$). The percent variance accounted for by each component is in parentheses. An ellipse is drawn around the data sets of pigs that did not receive ASP250. Black, day 0 (just prior to treatment); blue, day 8; pink, day 14. Circles, nonmedicated pigs; squares, medicated pigs.

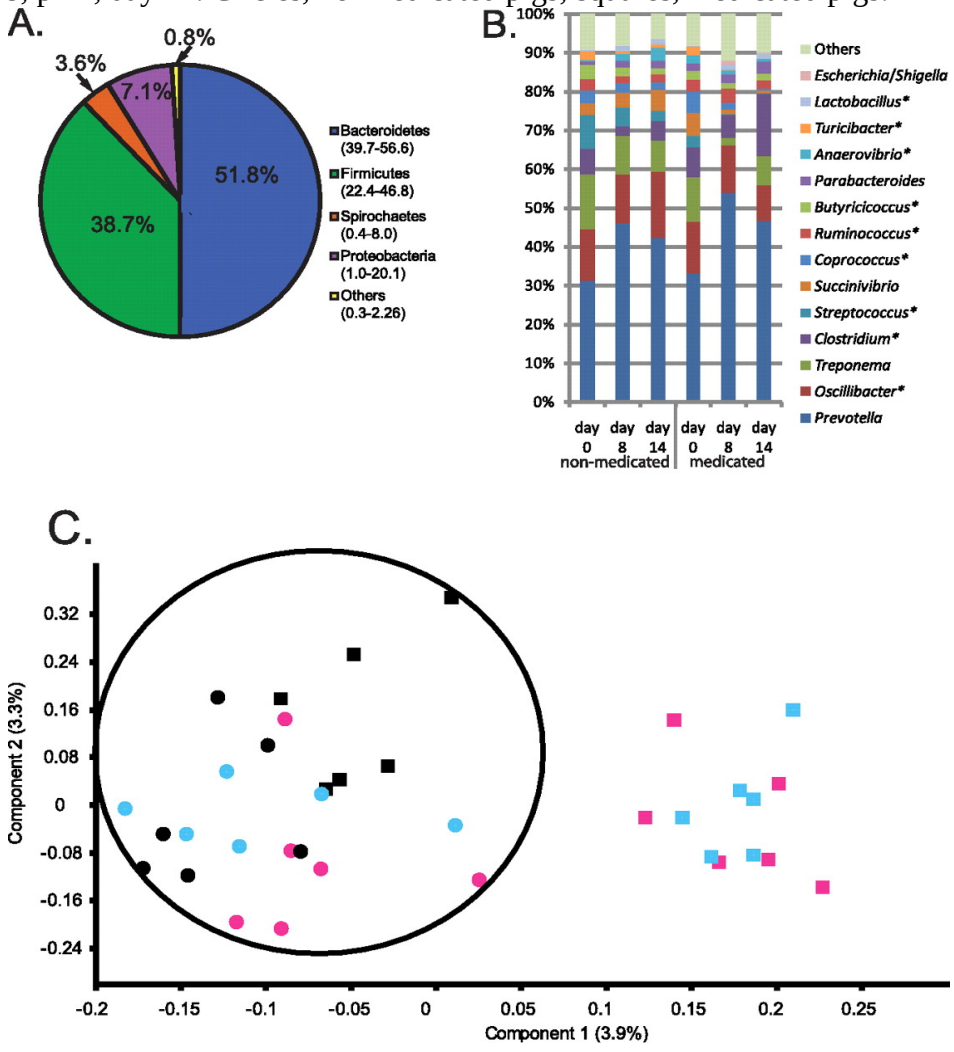


Figure C4. Population dynamics of bacteria and phages in swine fecal microbiomes. (A and C) Abundances of *Firmicutes* (A) and *Streptococcus* (C) bacteria and phages in the nonmedicated swine are plotted against time. (B and D) Regression analyses of the abundances of *Firmicutes* (B) and *Streptococcus* (D) phages against the respective bacterial abundances in all treatments and time points (r^2 = 0.21 and 0.23, respectively; $P < 0.1$ for both). In all figures, the bacterial abundances are pooled data from six animals.

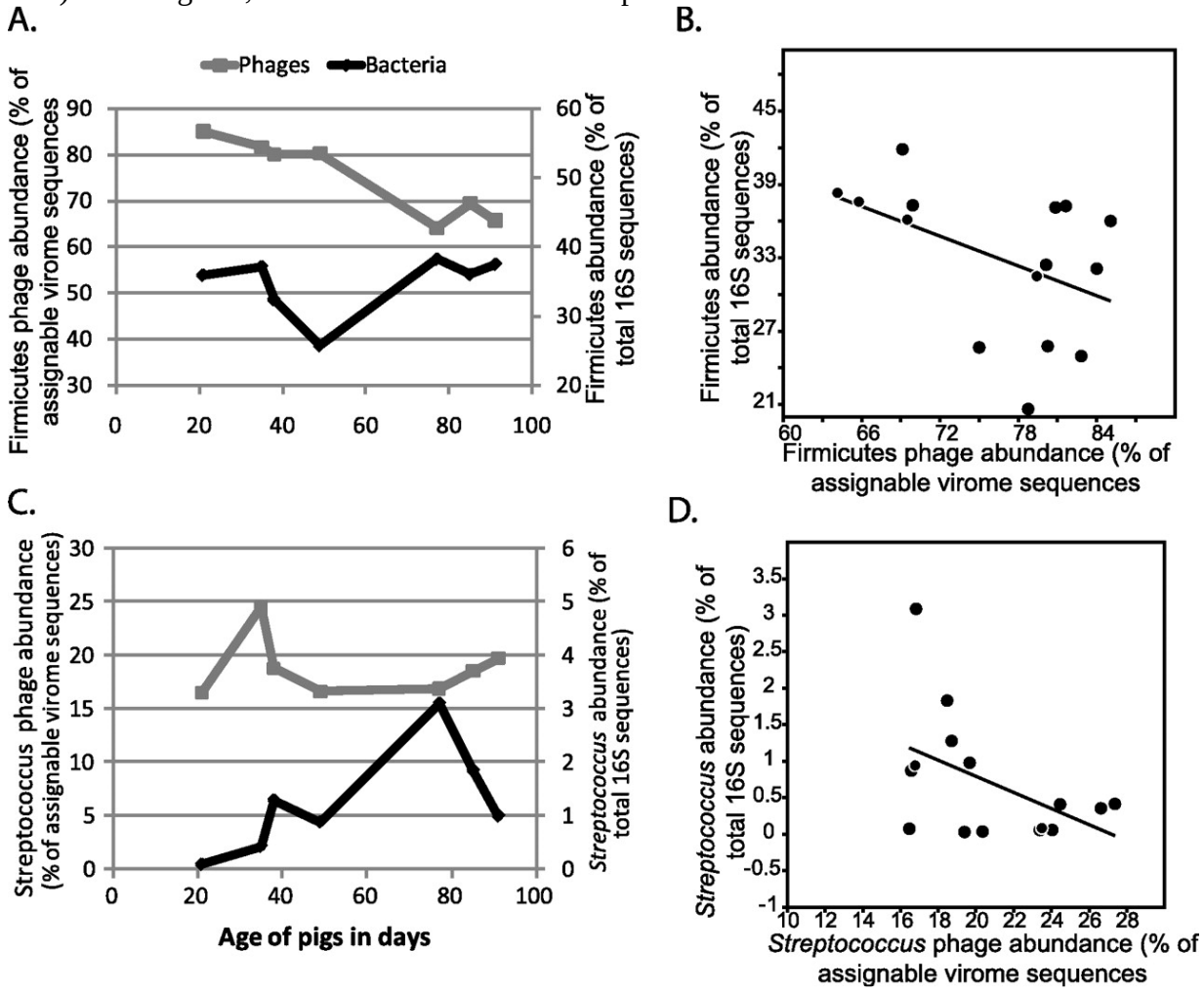


Figure C5. Box plot of integrase-encoding gene abundance in nonmedicated (n = 10) and medicated (n = 5) swine viromes ($P < 0.01$). Asterisks denote the means. The number of integrase-encoding genes was normalized by the total number of reads per virome.

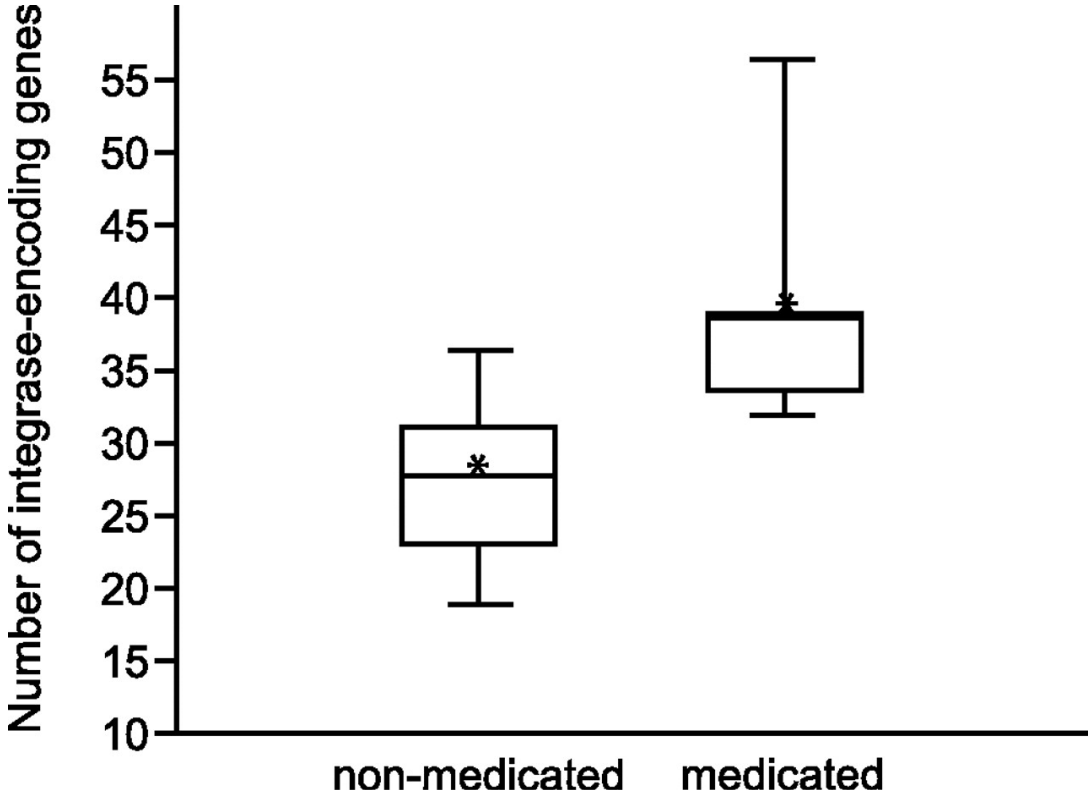


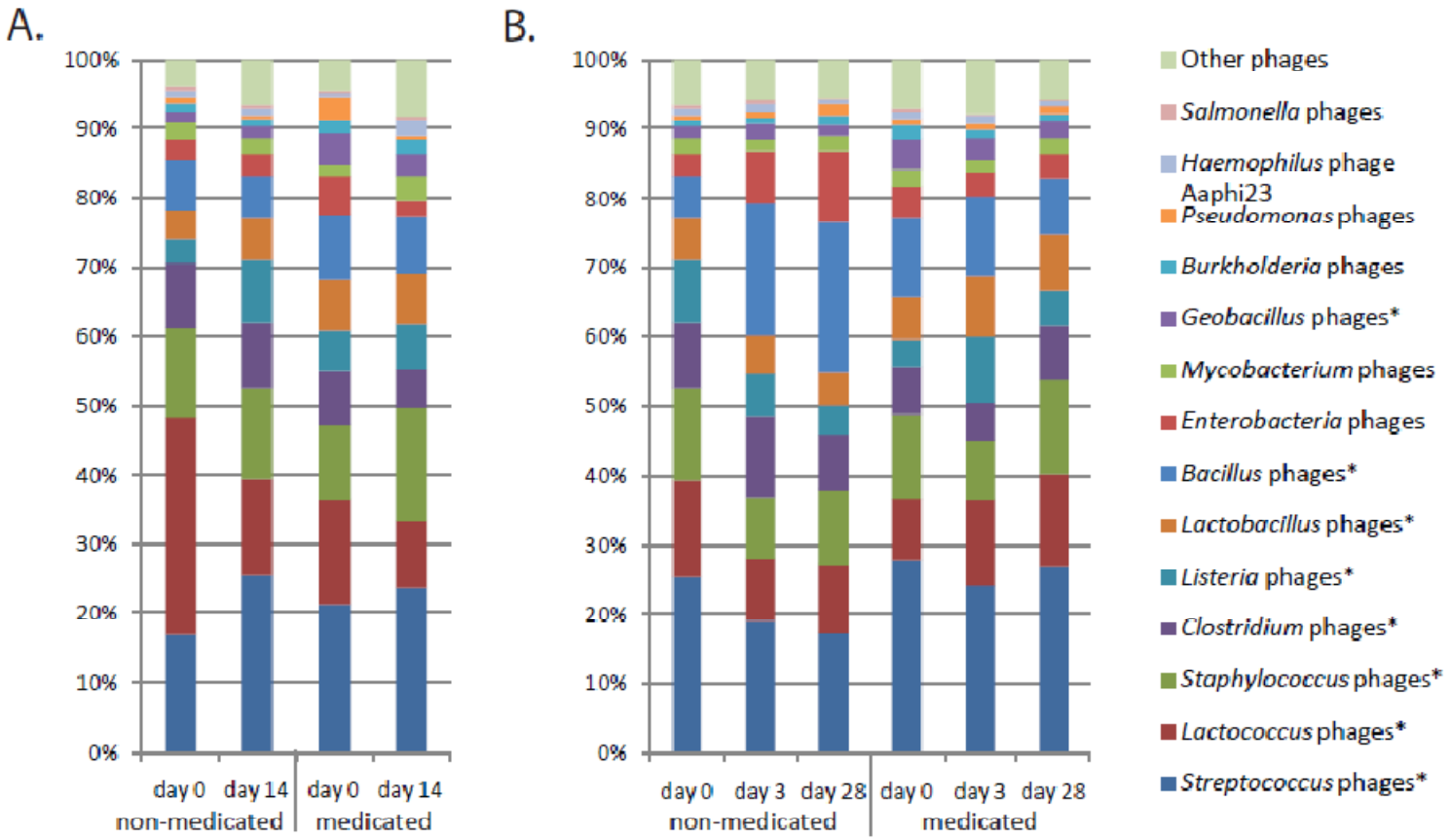
Table C1. Antibiotic resistance genes detected more than twice across all viromes, as annotated by the antibiotic resistance gene database (21)

Mechanism of resistance	Resistance gene	Resistance conferred by gene product	No. of hits to virome reads
Efflux pumps			
ABC transporter system	*bcrA*	Bacitracin	8
RND family transporter	*macB*	Macrolides	22
MFS family transporter	*mef*(A)	Macrolides	4
Target evasion			
Drug-insensitive dihydrofolate reductase	*dfrA*[a]	Trimethoprim	7
Ribosomal protection protein	*tet*(W)	Tetracycline	10
Modification of peptidylglycan biosynthetic pathway	*van*[b]	Vancomycin	26
Enzymatic deactivation			
NADPH-dependent oxidoreductase	*tet*37	Tetracycline	3

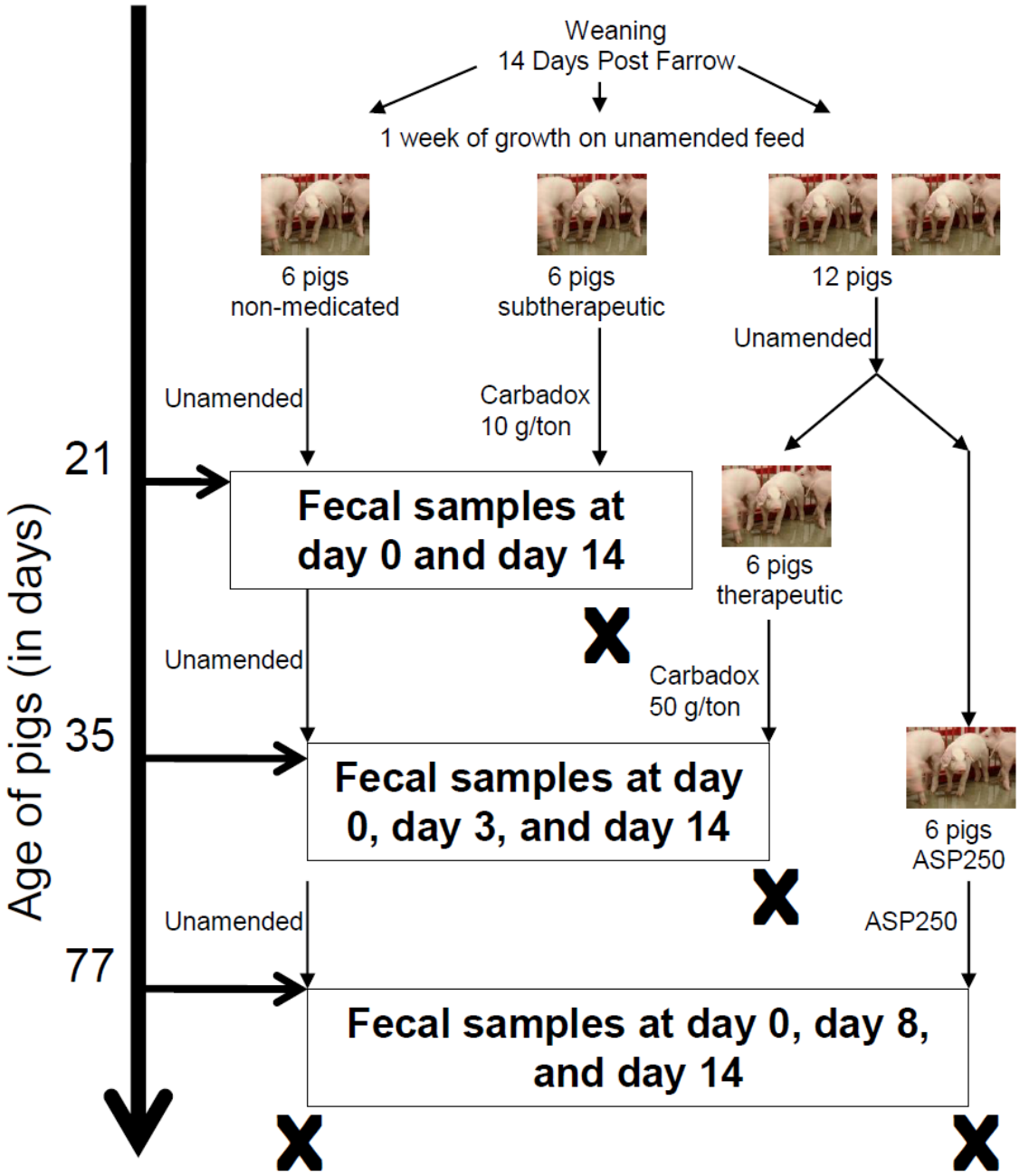
[a] The following DfrA-encoding genes were detected: *dfrA20*, *dfrA22*, *dfrA24*, and *dfrA26*.

[b] Portions (in parentheses) of the following vancomycin resistance pathways were detected in various viromes: VanA ($vanH_A$, $vanR_A$, $vanY_A$); VanB ($vanB$, $vanH_B$, $vanR_B$, $vanY_B$); VanC ($vanR_C$); VanD ($vanH_D$); VanE ($vanR_E$, $vanS_E$, $vanT_E$); VanG ($vanG$, $vanR_G$, $vanT_G$, $vanU_G$).

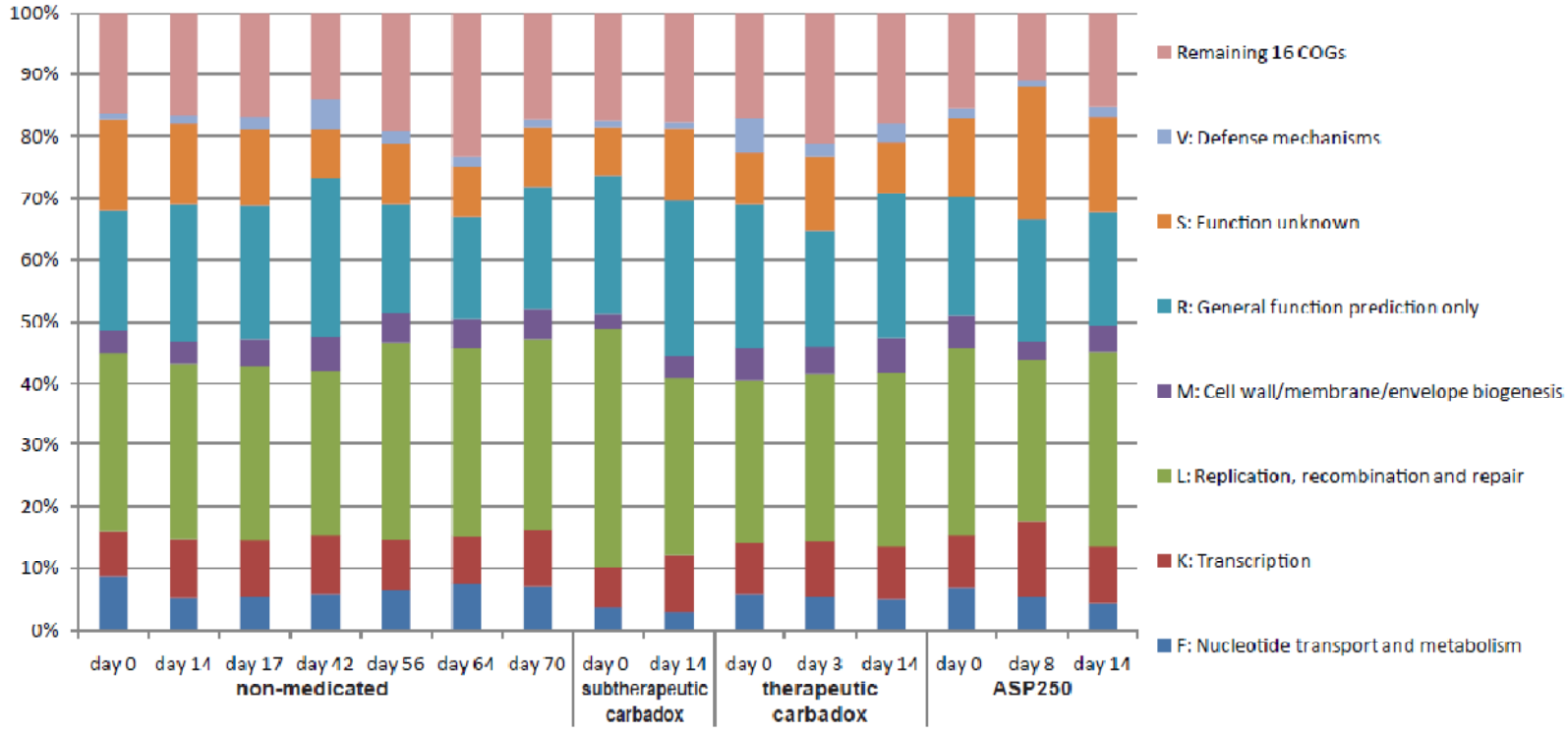
Supplemental Figure C1. GAAS was used to infer phylogeny of the phage-derived sequences from the subtherapeutic (A) and therapeutic (B) carbadox experiments. Phage taxa occurring at roughly <0.5% abundance are grouped as "Other phages." Asterisks denote those of the *Firmicutes* phylum. Note that because of the experimental design, nonmedicated day 14 in panel A is the same virome as nonmedicated day 0 in panel B.



Supplemental Figure C2. Schematic of swine fecal phage metagenomic study. Phages were isolated from fecal samples that were pooled from 6 pigs at each sampling point per treatment. Bacterial 16S rRNA genes were amplified and sequenced from individual pigs at each sampling point



Supplemental Figure C3. Clusters of orthologous groups (COGs) identified by CAMERA in assignable virome reads.



Supplemental Table C1. Mean and standard error (SE) of the most abundant bacteria in the feces of swine fed ASP250 and their nonmedicated counterparts.

	Non-medicated						Medicated					
	day 0		day 8		day 14		day 0		day 8		day 14	
Genus	mean	s.e.	mean	s.e.	mean	s.e.	mean	s.e.	mean	s.e.	mean	s.e.
Prevotella	30.02	3.90	44.01	5.21	39.36	5.07	32.10	4.16	52.76	5.59	46.78	5.13
Oscillibacter	13.37	1.80	12.51	1.25	17.46	3.49	13.09	1.98	12.58	5.20	9.54	0.86
Treponema	15.55	3.18	11.29	2.63	8.68	2.61	11.63	3.08	2.05	0.74	7.49	1.36
Clostridium	6.90	1.42	2.52	0.66	4.80	1.35	7.77	2.48	5.67	1.34	15.21	2.98
Streptococcus	7.99	2.68	4.88	1.33	2.49	0.72	3.28	1.64	0.28	0.19	0.08	0.06
Succinivibrio	2.74	1.11	3.86	1.19	4.56	2.26	5.25	1.41	1.11	0.29	0.64	0.25
Coprococcus	3.46	1.13	2.52	0.55	2.03	0.64	5.83	1.69	1.91	0.33	0.88	0.10
Ruminococcus	3.16	0.42	1.90	0.50	2.65	0.75	3.43	1.28	4.26	2.05	2.07	0.57
Butyricicoccus	3.50	0.56	2.47	0.61	1.78	0.38	2.40	0.68	1.46	0.50	1.70	0.41
Parabacteroides	0.81	0.11	1.91	0.50	2.47	0.52	1.84	0.66	2.61	0.96	3.29	1.01
Anaerovibrio	0.49	0.13	1.61	0.60	3.84	0.76	1.95	0.90	1.12	0.23	0.65	0.15
Turicibacter	2.42	0.64	0.82	0.11	0.80	0.13	2.23	0.49	0.06	0.03	0.05	0.02
Lactobacillus	0.15	0.06	1.37	0.35	1.64	0.66	0.23	0.10	1.10	0.15	1.41	0.40
Escherichia	0.07	0.02	0.01	0.01	0.00	0.00	0.12	0.02	1.11	0.47	0.32	0.16

Supplemental Table C2. Estimated operational taxonomic unit (OTU) diversity (± standard error) of 16S rRNA gene sequences in the ASP250 experiment, averaged within each sampling date and treatment.

Experimental group	Sampling day #						
	0	14	17	42	56	64	70
Non-medicated	599 ±165	238 ±25	394 ±44	275 ±27	848 ±234	671 ±107	636 ±134
Subtherapeutic carbadox	663 ±85[a]	648 ±238					
Therapeutic carbadox		307 ±27	497 ±121	336 ±23			
ASP250					1116 ±115	531 ±71	881 ±184

[a]Gray boxes indicate pre-medication sampling.

Supplemental Table C3. Summary of swine fecal phage metagenomic (virome) data.

	non-med day 0	**subther day 0**	**non-med day 14**	**subther day 14**	**ther day 0**	**non-med day 3**	**ther day 3**	**non-med day 28**	**ther day 28**	**non-med day 0**	**ASP250 day 0**	**non-med day 8**	**ASP250 day 8**	**non-med day 14**	**ASP250 day 14**
#reads (pre QC)[b]	25,980	91,801	122,762	13,971	60,047	97,408	38,480	65,791	53,872	64,264	102,989	84,947	101,259	176,197	118,923
#reads (post QC)	22,403	65,994	103,043	11,916	48,342	80,838	33,037	52,927	45,116	53,161	83,589	70,601	68,315	140,276	93,718
Average read length	358	385	359	374	378	375	363	366	363	344	345	335	356	375	357
#ARG[c] (%)	4 (.02)	3 (.005)	16 (.02)	0 (0)	12 (.02)	17 (.02)	13 (.04)	10 (.02)	7 (.02)	47 (.09)	56 (.07)	64 (.09)	7 (.01)	53 (.04)	28 (.03)

[a] Non-med, non-medicated; ther, therapeutic carbadox; subther, subtherapeutic carbadox.

[b]QC (quality control) was done using the 454 dereplicate filter (16).

[c]ARG, reads annotated as conferring resistance to antibiotics and toxic compounds, identified in the virulence, disease, and defense SEED subsystem (14).

Supplemental Table C4. Barcodes used in this study for parallel 16S rRNA gene sequencing.[a]

Barcode name	Sequence	Barcode name	Sequence
Wilmington-1	CACACACA	KingstonCA-31	TGCCGACA
BowlingGreen-2	CACAGTCA	AmmanCA-32	TAGGAACA
Munich-3	CACTCTCA	NairobiCA-33	CCGGCCCA
Fagaras-4	CACTGACA	SeoulCA-34	AACCTGCA
Cincinnati-5	CAGACTCA	RigaCA-35	TTCGTGCA
Cleveland-6	CAGAGACA	BeirutCA-36	AACACACA
Dayton-7	CAGTCACA	MaseruCA-37	TTCTTGCA
Bucharest-8	CAGTGTCA	VilniusCA-38	ACCTGACA
Brasov-9	CTCACTCA	BamakoCA-39	TTGACACA
Memphis-10	CTCAGACA	VallettaCA-40	TCCAGACA
Columbus-11	CTCTGTCA	MonacoCA-41	AAGGCCCA
Chatham -13	CTGACACA	WellingtonCA-42	ACGAGACA
Louisville-14	CTGAGTCA	ManaguaCA-43	TGGTGACA
St.Louis-15	CTGTCTCA	WarsawCA-44	TTCTCACA
Earlham-16	CTGTGACA	RiyadhCA-45	TTGAACCA
Desmoines-17	GACACTCA	BelgradeCA-46	CCGTTCCA
Iowacity-18	GACAGACA	SofiaCA-47	AAGCCCA
Albuquerque-19	GACTCACA	OttawaCA-48	CAAGACA
Houston-20	GACTGTCA	SantiagoCA-49	TATCACA
Madison-21	GAGACACA	BogotaCA-50	CGGTACA
Jacksonville-22	GAGAGTCA	ZagrebCA-51	AGACCCA
Chapelhill-23	GAGTCTCA	CopenhagenCA-52	AACCACA
Mankato-24	GAGTGACA	DjiboutiCA-53	TTCGACA
Siouxfalls-25	GTCACACA	SuvaCA-54	TAATCCA
Sandiego-26	GTCAGTCA	HelsinkiCA-55	TCACCCA
Hana-27	GTCTCTCA	ParisCA-56	TCCGCCA
Honolulu-28	GTCTGACA	AthensCA-57	ACGGCCA
Denver-29	GTGACTCA	DublinCA-58	AAGGCCA
Minneapolis-30	GTGAGACA		

[a]Barcodes 31-58 are derived from those of the Broad Institute (http://www.hmpdacc.org/doc/HMP_MDG_454_16S_Protocol_V4_2_102109.pdf).

APPENDIX D. TABLE OF MUCIN DEGRADERS ISOLATED FROM THE SWINE INTESTINAL TRACT.

Table D1. Identities of mucin-degrading bacteria, isolated from the swine intestinal tract. Taxonomic assignments of partial 16S rRNA gene sequences (V1-V3 region) were made using the Ribosomal Database project sequence classifier.

	Mucosa	Lumen	**Total**
Proteobacteria	67	37	**104**
Firmicutes	58	32	**90**
Bacteroidetes	26	64	**89**
Actinobacteria	24	16	**40**
Synergistetes	5	3	**8**
Total	**180**	**145**	**325**
Bacteroides	23	47	**70**
Escherichia	42	17	**59**
Enterococcus	28	14	**42**
Actinomyces	17	11	**28**
Eubacterium	8	6	**14**
Klebsiella	9	5	**14**
Parabacteroides	3	10	**13**
Clostridium	8	2	**10**
Enterobacter	8	0	**8**
Proteus	2	6	**8**
Cloacibacillus	5	3	**8**
Streptococcus	5	2	**7**
Citrobacter	4	3	**7**
Paraeggerthella	2	4	**6**
Desulfovibrio	0	5	**5**
Arcanobacterium	3	0	**3**
Finegoldia	2	0	**2**
Butyricicoccus	2	0	**2**
Atopobium	1	0	**1**
Collinsella	1	0	**1**
Lactococcus	1	0	**1**
Sharpea	0	1	**1**
Peptoniphilus	1	0	**1**
Peptostreptococcus	0	1	**1**
Sutterella	1	0	**1**
unclassified *Lachnospiraceae*	0	4	**4**
unclassified *Ruminococcaceae*	2	1	**3**
unclassified *Enterobacteriaceae*	1	1	**2**
unclassified *Coriobacteriaceae*	0	1	**1**
unclassified *Clostridiales*	0	1	**1**
unclassified *Eubacteriaceae*	1	0	**1**